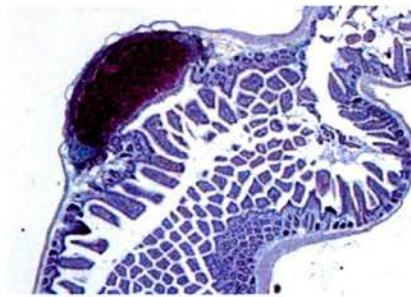


# **The role of draining lymphoid tissues in TSE agent neuroinvasion**

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University of Edinburgh for the degree of Doctor of Philosophy**

The programme of research was carried out at the Institute for Animal Health,  
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## **Declaration**

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors Dr. Neil Mabbott, Prof. Moira Bruce and Prof. John Hopkins, unless otherwise stated. No part of this work has been, or will be submitted for any other degree, or professional qualification.

Bridget R. Glaysher

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## **Definitions of abbreviations**

<b>Abbreviation</b>	<b>Definition</b>
(x) <sup>-/-</sup>	Mice homozygous for deficiency of a particular gene where (x) represents the gene of interest
(x) <sup>+/+</sup>	Mice homozygous for the presence of a particular gene where (x) represents the gene of interest
μMT	Immunoglobulin M transmembrane exon
AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
BSE	Bovine spongiform encephalopathy
C1q	Complement component 1q
C2	Complement component 2
C3	Complement component 3
C5	Complement component 5
C9	Complement component 9
CD	Cluster of differentiation
CJD	Creutzfeldt-Jakob disease
CLN	Cervical lymph node
CMLN	Cranial mediastinal lymph node
CNS	Central nervous system
CR1	Complement receptor 1
CR2	Complement receptor 2
CWD	Chronic wasting disease
CXCL13	CXC ligand 13
CXCR5	CXC receptor 5
DAB	Diaminobenzidine
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbant assay
fCJD	Familial Creutzfeldt-Jakob disease
FDC	Follicular dendritic cell
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
GALT	Gut-associated lymphoid tissue
GFAP	Glial fibrillary acidic protein
GSS	Gerstmann-Straussler-Scheinker syndrome
H & E	Haematoxylin and eosin
i.c.	Intra-cranial
ICAM-1	Intercellular adhesion molecule-1
iCJD	Iatrogenic Creutzfeldt-Jakob disease
Id2	Inhibitor of DNA binding 2
IgA	Immunoglobulin A
IgG	Immunoglobulin G
iILF	Immature isolated lymphoid follicle
IKKα	Inhibitor of NFκB kinase α subunit
IKKβ	Inhibitor of NFκB kinase β subunit
IL-7	Interleukin -7
ILF	Isolated lymphoid follicle
ILN	Inguinal lymph node
i.p.	Intra-peritoneal
kDa	Kilodaltons
LN	Lymph node
LT	Lymphotoxin
LTα	Lymphotoxin -α
LTα <sub>1</sub> β <sub>2</sub>	Lymphotoxin heterotrimer



LT $\alpha_3$	Lymphotoxin- $\alpha$ homotrimer
LT $\beta$	Lymphotoxin - $\beta$
LT $\beta$ R	Lymphotoxin - $\beta$ receptor
LT $\beta$ R-Ig	Fusion protein of LT $\beta$ R linked to the Fc portion of human IgG
LTIC	Lymphoid tissue initiating cell
MAC	Membrane attack complex
mAdCAM-1	Mucosal addressin cell adhesion molecule-1
mILF	Mature isolated lymphoid follicle
MLN	Mesenteric lymph node
NALT	Nasal-associated lymphoid tissue
NaPTA	Sodium phosphotungstic acid
NF $\kappa$ B	Nuclear factor $\kappa$ B
NIK	NF $\kappa$ B inducing kinase
NK	Natural killer
PK	Proteinase K
PLP	Periodate-lysine-paraformaldehyde
PNA	Peanut agglutinin
PP	Peyer's patch
<i>Prnp</i>	Gene encoding the prion protein in mice
<i>PRNP</i>	Gene encoding the prion protein in humans
<i>PrP</i>	Gene encoding the prion protein in animals other than humans and mice
PrP <sup>c</sup>	Cellular (normal) form of the prion protein
PrP <sup>d</sup>	Disease specific form of the prion protein
PrP <sup>Sc</sup>	Disease specific form of the prion protein where resistance to PK has been confirmed
ROR $\gamma$	Retinoid related orphan receptor $\gamma$
SCID	Severe combined immunodeficiency
sCJD	Sporadic Creutzfeldt-Jakob disease
S.E.M.	Standard error of the mean
SILT	Solitary intestinal lymphoid tissue
SRBC	Sheep red blood cell
TME	Transmissible mink encephalopathy
TNF $\alpha$	Tumour necrosis factor - $\alpha$
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TRAF	TNF associated factor
TRANCE	TNF-related activation-induced cytokine
TSE	Transmissible spongiform encephalopathy
UEA-1	<i>Ulex europaeus</i> agglutinin -1
VCAM-1	Vascular cell adhesion molecule-1
vCJD	Variant Creutzfeldt-Jakob disease
WT	Wild-type
WT->LT $\alpha^{-/-}$	LT $\alpha^{-/-}$ mice reconstituted with WT bone marrow
WT->LT $\beta^{-/-}$	LT $\beta^{-/-}$ mice reconstituted with WT bone marrow
WT->WT	WT mice reconstituted with WT bone marrow

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## **Abstract**

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases. The causative agent for these diseases is unknown. After peripheral inoculation, accumulation and replication of the agent has been found to occur in lymphoid tissues prior to spreading to the CNS and brain where pathology is caused. Within lymphoid tissues, follicular dendritic cells (FDCs) are critical for the accumulation of the TSE agent. However, specifically which lymphoid tissues neuroinvasion occurs from is unclear. The aims of this thesis were to determine the importance of draining lymphoid tissue in pathogenesis of the ME7 strain of scrapie agent after inoculation via the oral, scarification or intra-peritoneal (i.p.) routes. The formation of most lymphoid tissue is critically dependent on lymphotoxin (LT)  $\alpha\beta$  signalling through LT $\beta$ R during gestation. Mice in which this signalling has been interrupted, either through *in utero* treatment with LT $\beta$ R-Ig or by genetic deficiency of LT $\alpha$  or LT $\beta$ , lack various lymphoid tissues. LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice also lack FDCs so these were reconstituted with wild-type (WT) bone marrow, thus restoring FDCs without inducing the formation of missing lymphoid tissues. After oral inoculation Peyer's patches (PPs) within the intestine were found to be crucial for pathogenesis as mice lacking PPs did not develop disease. Isolated lymphoid follicles (ILFs) which resemble PPs have recently been described in the murine small intestine and are thought to compensate for PP-deficiency. ILFs were investigated here and found to contain FDCs and, as a result support neuroinvasion of the scrapie agent in the absence of PPs. Mice lacking inguinal lymph nodes (ILNs) displayed decreased susceptibility to scrapie disease after inoculation via scarification of the

skin of the thigh. This demonstrated that the ILNs are important, but neuroinvasion could occur from other tissues in some cases. The cranial mediastinal lymph nodes (CMLNs) drain the peritoneal cavity. Investigations in this thesis revealed both  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack these nodes.  $LT\alpha^{-/-}$  mice were less susceptible and had delayed onset of disease compared to WT mice whereas  $LT\beta^{-/-}$  mice were as susceptible and developed disease in the same timeframe as WT mice after i.p. inoculation. This demonstrated that the CMLN is dispensable for neuroinvasion via the i.p. route, but raised the possibility that lymphoid tissue present in  $LT\beta^{-/-}$  mice and absent from  $LT\alpha^{-/-}$  mice was important in supporting neuroinvasion. In all three routes the lack of draining lymphoid tissue had an effect on the pathogenesis of disease demonstrating the importance of these tissues in the peripheral pathogenesis of TSEs.

# 1

## Introduction

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# **1. Introduction**

## **1.1. Transmissible spongiform encephalopathies**

### **1.1.1 TSE diseases**

Transmissible spongiform encephalopathies (TSEs) or “prion” diseases are a group of invariably fatal, neurodegenerative diseases which affect both humans and domestic and wild animals. They have also been described as slow viruses, subacute spongiform encephalopathies and transmissible dementias (Collinge, 2001).

The oldest known TSE disease is scrapie, a disease of sheep and goats which was first recognised about 300 years ago (McGowan, 1922). This disease is named for the clinical signs it produces in affected animals (tremours to the head and neck, scratching, rubbing, and gait abnormalities; McGowan, 1922) and is also known as “la tremblante” (French for trembling), “traberkrankheit” (German for trotting disease) or “rida” (Icelandic for ataxia or tremour) in other countries (Detwiler *et al.*, 2000). Scrapie is endemic in most sheep producing countries worldwide, with the exception of Australia which has not reported any cases of scrapie disease in its national flock. Scrapie was originally thought to be a genetic disease (McGowan, 1922), but an outbreak of scrapie due to a vaccine composed of scrapie-contaminated spleen and brain tissue first showed that the disease was infectious (Gordon, 1946). An early experiment demonstrated that scrapie could be transmitted between sheep and goats (Cuille & Chelle, 1936), subsequently the similarities in the epidemiology, pathology and clinical signs between scrapie in sheep and TSEs of other species were noted (Hadlow, 1959). The route of transmission of scrapie is unclear, but may



involve both horizontal (sheep to sheep; Brotherston *et al.*, 1968) and vertical (ewe to lamb) transmission (Foster *et al.*, 1996).

TSEs in other species have been described - such as chronic wasting disease (CWD) a TSE which affects mule deer (*Odocoileus hemionus hemionus*) and elk (*Cervus elephus nelsoni*; Williams & Young, 1980) transmissible mink encephalopathy (TME) that affects farmed mink (Hartsough & Berger, 1965) and familial fatal insomnia (FFI; Medori *et al.*, 1992), Gerstmann Straussler Scheinker syndrome (GSS; Gerstmann *et al.*, 1936) and Creutzfeldt-Jakob disease (CJD; Creutzfeldt, 1920; Jakob, 1921a, 1921b) that affect humans. Table 1.1 describes the nomenclature and aetiology of many TSE diseases.

In the early 1980s an outbreak of TSE disease in cattle (which were not previously known to be natural hosts of TSE disease) led to the first description of bovine spongiform encephalopathy (BSE; Wells *et al.*, 1987). This outbreak is thought to have arisen by TSE contaminated protein derived from rendered carcasses of ruminants being used to supplement the diet of intensively farmed cattle (Anderson *et al.*, 1996; Wilesmith *et al.*, 1991). The original source of the contaminant and the conditions which allowed the emergence of BSE remain unclear (Collee & Bradley, 1997a, 1997b). The wide use of cattle products in feedstuffs resulted in subsequent outbreaks of feline spongiform encephalopathy (FSE) in domestic and exotic cats (Wells *et al.*, 1987; Wyatt *et al.*, 1991), and TSE diseases in other exotic animals such as kudu and oryx (Jeffrey & Wells, 1988; Kirkwood *et al.*, 1990; Wells *et al.*, 1987). More importantly from a public health point of view an outbreak of a variant

Disease	Affected species	Aetiology	Reference
Scrapie	Sheep and goats	Acquired via unknown route	(McGowan, 1922)
Chronic Wasting Disease (CWD)	Mule deer and elk	Acquired via unknown route	(Williams & Young, 1980)
Transmissible Mink Encephalopathy (TME)	Mink	Acquired via unknown route	(Hartsough & Berger, 1965)
Familial Creutzfeldt-Jakob Disease (fCJD)	Humans	Inherited (associated with <i>PRNP</i> mutations)	(Goldfarb <i>et al.</i> , 1992)
Sporadic Creutzfeldt-Jakob Disease (sCJD)	Humans	Unclear; Somatic <i>PRNP</i> mutation or spontaneous PrP conversion	(Creutzfeldt, 1920; Jakob, 1921a, 1921b)
Iatrogenic Creutzfeldt-Jakob Disease (iCJD)	Humans	Acquired via accidental medical exposure	(Duffy <i>et al.</i> , 1974)
Variant Creutzfeldt-Jakob Disease (vCJD)	Humans	Acquired via dietary exposure to BSE contaminated meat	(Bruce <i>et al.</i> , 1997; Will <i>et al.</i> , 1996)
Gerstmann-Straussler-Scheinker syndrome	Human	Inherited (associated with <i>PRNP</i> mutations)	(Gerstmann <i>et al.</i> , 1936)
Fatal Familial Insomnia (FFI)	Human	Inherited (associated with <i>PRNP</i> mutations)	(Medori <i>et al.</i> , 1992)
Bovine Spongiform Encephalopathy (BSE)	Cattle	Acquired via dietary exposure to BSE contaminated feed	(Wells <i>et al.</i> , 1987)
Feline Spongiform Encephalopathy (FSE)	Domestic and exotic cats	Acquired via dietary exposure to BSE contaminated meat	(Wells <i>et al.</i> , 1987)
Exotic TSEs	Ungulates	Acquired via dietary exposure to BSE contaminated feed	(Bruce <i>et al.</i> , 1997; Wells <i>et al.</i> , 1987)

**Table 1.1- Transmissible spongiform encephalopathies of animals and humans – nomenclature and aetiology.**

of the human Creutzfeldt-Jakob disease (vCJD) has been shown to be caused by the same agent as the BSE epidemic (Bruce *et al.*, 1997; Hill *et al.*, 1997) and therefore is presumed to have arisen and spread by the consumption of BSE contaminated beef and other cattle derived products. Figures to the 6<sup>th</sup> November 2006 show there have been 158 deaths from vCJD in the UK and a further 6 patients living with the clinical signs of vCJD (figures from <http://www.cjd.ed.ac.uk/figures.htm>). The BSE epidemic and the subsequent transmission of the BSE agent across the species barrier to humans has led to vast scientific and medical interest in these diseases with a view to developing both diagnostic strategies to identify them and therapeutic strategies to control them.

### **1.1.2 The nature of the causative TSE agent**

The nature of the causative TSE agent is unclear and is a hotly debated topic among TSE researchers. At present there are at least three main theories as to the precise nature of the causative agent.

Historically, TSEs were thought to be slow viruses due to the long incubation time between exposure to the infectious agent and the onset of clinical signs of disease (Collinge, 2001). The “virus hypothesis” proposes that the TSE agent is a virus as TSEs are transmissible, display strain specific variation and initially virus particles were thought to have been identified (Cho, 1976; Cho & Greig, 1975). However, this hypothesis now seems unlikely as treatments that would inactivate viral replication such as exposure to ultraviolet light, ionising radiation, dry heat or chemical treatments do not affect the pathogenesis of TSEs. Additionally, TSE



disease specific nucleic acid has never been identified (Alper *et al.*, 1966; Brown *et al.*, 1986; Kimberlin *et al.*, 1983; Somerville *et al.*, 1997a; Taylor *et al.*, 1996a).

It has been suggested that it is thermodynamically possible for a protein to be the only component of an infectious agent; consequently it was proposed that TSE agent could consist solely of a protein (Griffiths, 1967). Subsequently, a relatively proteinase resistant host protein was found to co-purify with infectivity leading to the proposal of what is known as the “prion hypothesis” (Bolton *et al.*, 1982; Prusiner, 1982). The term “prion” was coined from proteinaceous infectious particle and the prion hypothesis is, therefore, the idea that the relatively proteinase resistant, detergent insoluble, misfolded form of the prion protein (termed PrP<sup>Sc</sup>; Meyer *et al.*, 1986) is the major, or sole component of the infectious agent (Prusiner, 1982). TSE disease is propagated by PrP<sup>Sc</sup> converting the normal cellular form of the prion protein (PrP<sup>C</sup>) into PrP<sup>Sc</sup>. This hypothesis explains why disease specific nucleic acid has never been identified, and why viral inactivation treatments do not affect TSE pathogenesis. This hypothesis is further supported by evidence suggesting that prions created *in vitro* can be infectious when used *in vivo* (Legname *et al.*, 2004) and that PrP<sup>Sc</sup> can convert PrP<sup>C</sup> to PrP<sup>Sc</sup> *in vitro* (Kocisko *et al.*, 1994). However, the prion hypothesis does not explain the existence of TSE strains. It has been proposed that strains are due to different conformations of PrP<sup>Sc</sup> (Prusiner, 1991) or different glycosylation patterns (Ermonval *et al.*, 2003). However, different conformations or glycosylation patterns have not been shown to be the cause of disease (Soto & Castilla, 2004). The demonstration of TSE infectivity in the absence of the misfolded form of the prion protein (Barron *et al.*, 2001; Lasmezas *et al.*,

1997) and presence of PrP<sup>Sc</sup> in the absence of TSE infectivity are also not readily explained by the prion hypothesis (Prusiner *et al.*, 1990).

This has led to the proposal of the “virino hypothesis”. This is the suggestion that the TSE agent consists of an informational component (such as nucleic acid) that interacts with host PrP<sup>C</sup> (Dickinson & Outram, 1988). The conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> may prevent the degradation of the informational component (Farquhar *et al.*, 1998). As this agent would not have to encode the synthesis of viral coat proteins a piece of nucleic acid involved in this process could be very small. Thus this hypothesis explains the presence of TSE strains while allowing for the absence of detectable nucleic acid. This hypothesis could also overcome the difficulty of strains as the informational component may have the ability to confer strain specific characteristics.

### **1.1.3 The cellular form of the prion protein**

Whatever the nature of the TSE agent it is clear that the prion protein is central to TSE disease as the misfolded form co-purifies with infectivity in all cases of natural disease and mice genetically deficient in PrP are resistant to disease (Bueler *et al.*, 1993; Manson *et al.*, 1994a; Sakaguchi *et al.*, 1995). This has led to considerable interest in the structure and function of PrP<sup>C</sup>. PrP<sup>C</sup> is encoded a single gene in the genome of rodents, ruminants and man (Oesch *et al.*, 1985; Westaway & Prusiner, 1986). This gene is denoted *Prnp* in mice, the equivalent gene is denoted *PRNP* in humans and *PrP* in other species. PrP<sup>C</sup> is a sialoglycoprotein made up of approximately 210 amino acid residues and has several distinct domains that may be

related to function, including a minimum of four copper binding octapeptide repeats, a glycosphosphatidylinositol (GPI) anchor and an N-terminal signal peptide. There are two sites on the PrP<sup>C</sup> molecule to which a carbohydrate may be added during synthesis. These are asparagine residues at positions 181 and 197. The differential glycosylation of these site results in three forms of PrP<sup>C</sup> – unglycosylated, monoglycosylated (where a sugar molecule is added at either one of these sites) or diglycosylated (where a sugar molecule is added at both sites; Oesch *et al.*, 1985).

PrP<sup>C</sup> is expressed at the highest level in the tissues of the central and peripheral nervous systems (Kretzschmar *et al.*, 1986; Oesch *et al.*, 1985). Lower levels of expression of PrP<sup>C</sup> are found in other peripheral tissues including lymphoid tissues (Caughey *et al.*, 1988; Dodelet & Cashman, 1998; Mabbott *et al.*, 1997). The biological function of this molecule is unknown. Three independent PrP<sup>-/-</sup> mice lines have been generated (Bueler *et al.*, 1992; Manson *et al.*, 1994a; Sakaguchi *et al.*, 1995). These develop normally and appear healthy, however, subtle abnormalities have been reported in these mice such as disturbances in circadian rhythms (Tobler *et al.*, 1996) and impaired T lymphocyte responses (Kubosaki *et al.*, 2003; Lewicki *et al.*, 2003; Mabbott *et al.*, 1997). Experiments have suggested that PrP<sup>C</sup> may have a role in preventing apoptosis of both human and murine neuronal cell lines (Bounhar *et al.*, 2001; Kim *et al.*, 2004; Kuwhara *et al.*, 1999; Roucou *et al.*, 2004). This hypothesis is further supported by the finding that after cross-linking PrP<sup>C</sup> with a PrP specific antibody *in vivo* and thus preventing PrP<sup>C</sup> from functioning normally mass apoptosis of neurones was observed (Solforosi *et al.*, 2004). Other evidence has suggested that function of PrP<sup>C</sup> may be to prevent neuronal death due to copper



toxicity as PrP<sup>C</sup> can bind copper (Brown, 2004; Brown *et al.*, 1997a; Brown *et al.*, 1997b; Millhauser, 2004). Markers of oxidative stress are found at increased levels in the brains of PrP<sup>-/-</sup> mice (Wong *et al.*, 2001) and mice infected with scrapie display poor responses to oxidative stress (Milhabet *et al.*, 2000) suggesting that PrP<sup>C</sup> may play a role in the prevention of damage to neurones due to oxidative stress by acting as a superoxide dismutase (Brown *et al.*, 1999a). PrP<sup>C</sup> may also have a function in signal transduction by affecting the activity of the tyrosine kinase Fyn (Mouillet-Richard *et al.*, 2000).

Although the function of PrP<sup>C</sup> is unknown, the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> seems to be vital for the establishment of TSE disease as in the absence of PrP<sup>C</sup> disease does not develop (Bueler *et al.*, 1993; Manson *et al.*, 1994a; Sakaguchi *et al.*, 1995) and PrP<sup>Sc</sup> co-purifies with infectivity in natural cases of TSE disease (Bolton *et al.*, 1982; Meyer *et al.*, 1986). When PrP<sup>+/+</sup> neurones were grafted into the brains of PrP<sup>-/-</sup> mice disease specific pathology was observed only in the grafted tissue suggesting that the expression of PrP<sup>C</sup> is vital for the generation of pathology (Brandner *et al.*, 1996). Experiments have also showed that heterozygous PrP<sup>+/-</sup> mice take longer to develop disease than PrP<sup>+/+</sup> counterparts (Manson *et al.*, 1994b) again demonstrating the importance of PrP<sup>C</sup> for the development of TSE disease.

The survival time of mice inoculated with scrapie is influenced by polymorphisms in the gene encoding PrP<sup>C</sup>. Initially these were described as a separate gene termed *Sinc* for scrapie incubation period (Dickinson *et al.*, 1968), which has two alleles, *s7* and *p7*, for short and prolonged incubation periods respectively (Dickinson &

MacKay, 1961; Dickinson & Meikle, 1971; Dickinson *et al.*, 1968). Later research revealed that these correlated to polymorphisms at codons 108 and 189 of the *Prnp* gene with Leu-108 and Thr-189 corresponding to *Sinc*<sup>s7</sup> and Phe-108 and Val-189 corresponding to *Sinc*<sup>p7</sup> (Moore *et al.*, 1998). These alleles are now termed *Prnp*<sup>a</sup> and *Prnp*<sup>b</sup> respectively. More recently a third allele (*Prnp*<sup>c</sup>) has been identified where Phe-108 and Thr-189 also influences the incubation period of scrapie in mice (Lloyd *et al.*, 2004).

Polymorphisms in the gene encoding PrP<sup>C</sup> have also been observed to have an effect on the pathogenesis of natural TSEs. For example, in sheep, polymorphisms at codons 136, 154 and 171 in the ovine *PrP* gene have been shown to influence susceptibility to natural scrapie. Depending on the allelic combination and the breed of sheep these polymorphisms can lead to susceptibility or resistance to disease. For example in the Suffolk breed sheep homozygous for polymorphisms Ala-136, Arg-154 and Gln-171 (ARQ/ARQ genotype) are highly susceptible, whereas sheep with the genotype ARR/ARR are resistant. In Cheviot sheep the ARQ/ARQ genotype is associated with resistance while VRQ/VRQ sheep are highly susceptible (Goldmann *et al.*, 1991; Goldmann *et al.*, 1994; Hunter, 1998).

Similarly, polymorphisms at codon 129 in the *PRNP* gene of humans influence susceptibility to vCJD – all clinical vCJD cases have been found to be methionine homozygotes at this codon (Ironside, 2000). Additionally, mutations in the *PRNP* gene, such as P102L, D178N and E200K are each closely associated with the

development of GSS, FFI and fCJD respectively (Hsaio *et al.*, 1991; Hsaio *et al.*, 1989; Medori *et al.*, 1992).

#### **1.1.4 Distinguishing the cellular form from the disease specific form of the prion protein**

PrP<sup>Sc</sup> has some properties which allow it to be distinguished from PrP<sup>C</sup> such as proteinase resistance and insolubility when treated with detergents. When PrP<sup>Sc</sup> is treated with proteinase K (PK) the 33-35 kDa protein is truncated to leave a 20-30 kDa proteinase resistant core. When PrP<sup>C</sup> is treated similarly it is fully hydrolysed. Thus when PK treated tissue homogenates are analysed by immunoblotting PrP will be detected only in infected samples (Oesch *et al.*, 1985). Immunoblotting of either PrP<sup>C</sup> or PrP<sup>Sc</sup> results in a three band pattern, the three bands representing the unglycosylated, monoglycosylated and diglycosylated forms of the protein (in order of increasing molecular mass; Oesch *et al.*, 1985).

The relative resistance of PrP<sup>Sc</sup> to PK also allows PrP<sup>Sc</sup> to be distinguished from PrP<sup>C</sup> on tissue sections. Tissue sections can be fixed to nitrocellulose, treated with PK followed by immunostaining with PrP specific antibodies. Thus only PrP<sup>Sc</sup> will be left on the nitrocellulose for detection (Schulz-Schaeffer *et al.*, 2000). The absence of a commercially available antibody to distinguish between PrP<sup>C</sup> and PrP<sup>Sc</sup> makes conventional immunostaining and other techniques that do not allow for PK digestion difficult. PrP<sup>Sc</sup> specific antibodies have recently been generated which may make analysis of future TSE pathogenesis experiments easier to interpret (Paramithiotis *et al.*, 2003). In this thesis, disease specific accumulations of the PrP



have been visualised using conventional immunostaining with the monoclonal antiserum 1B3 as a series of studies have revealed these accumulations to occur only in the tissues of TSE affected animals and to correlate closely with the presence of the TSE agent and the detection of disease specific PrP is enhanced by using periodate-lysine-paraformaldehyde (PLP) fixative and pre-treatment with formic acid (see section 2.4.2; Beekes & McBride, 2000; Brown *et al.*, 1999b; Mabbott *et al.*, 2000a; Mabbott *et al.*, 2003; McBride *et al.*, 1992; McBride *et al.*, 2001). In this case, where PK resistance has not been confirmed, these accumulations are referred to as PrP<sup>d</sup> to distinguish them from PK resistant PrP<sup>Sc</sup>. The use of conventional immunostaining has the advantage that counterstaining of the tissue may be done allowing for the anatomical location of PrP<sup>d</sup> accumulations to be ascertained. This cannot be done where treatment has digested the tissue away.

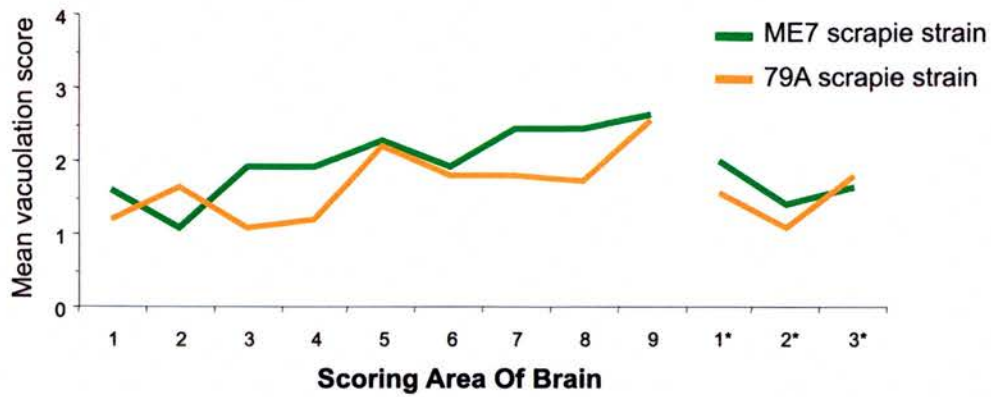
PrP<sup>Sc</sup> can also be distinguished from PrP<sup>C</sup> based on its misfolded conformation. Infrared and circular dichroism experiments showed that PrP<sup>C</sup> consists of approximately 43 %  $\alpha$ -helix and 3 %  $\beta$ -sheets whereas PrP<sup>Sc</sup> consists of 34 %  $\alpha$ -helix and 43 %  $\beta$ -sheet (Pan *et al.*, 1993). Large amounts of material are required to do this kind of analysis making it impossible for this to be carried out on tissues from individual mice.

### **1.1.5 Neuropathological characterisation of TSE strains in rodent models**

At present around twenty strains of scrapie have been identified by passage in lines of inbred mice. TSE strains are distinguished according to the disease incubation period and the vacuolar pathology in the brain. When a serially passaged scrapie strain is injected at a high dose directly into the brains of a group of mice of the same line the incubation periods are highly reproducible such that the standard error is less than 2 % of the mean (Dickinson & Meikle, 1971; Dickinson *et al.*, 1968). However, two different strains of the scrapie agent in the same line of mice will result in two distinct incubation periods as will the same scrapie agent in two different lines of mice. Thus measuring the disease incubation period can be used to distinguish scrapie strains. Similarly, the pattern of vacuolation in the brain can also be used to distinguish scrapie strains. Brain areas are identified and scored according to the severity and distribution of the vacuolation. These values can be plotted on a graph to generate what is termed a “lesion profile” (Fig 1.1). Mice from the same line infected with the same strain of scrapie agent will repeatedly result in similar lesion profiles, whereas the same strain of agent injected into a different line of mice will result in a different lesion profile. Similarly, two different strains of the agent injected into the same line of mice will result in two distinct lesion profiles (Fig 1.1; Fraser & Dickinson, 1973).

Glycoform analysis can also be used to distinguish TSE strains. This uses the intensity and migration of the PrP glycoform banding pattern obtained using immunoblotting. Different strains have different ratios of the three glycosylation states of PrP. These migrate at different speeds when subjected to gel





**Figure 1.1- Vacuolar pathology of scrapie strains ME7 and 79A.** Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (1-9) or white matter (1\*-3\*) areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septum; 8, retrosplenial and adjacent motor cortex; 9, cingulate and adjacent motor cortex; 1\*, inferior and middle cerebellar peduncles; 2\*, decussation of the superior peduncles; 3\*, cerebral peduncles. Each point represents mean vacuolation score.

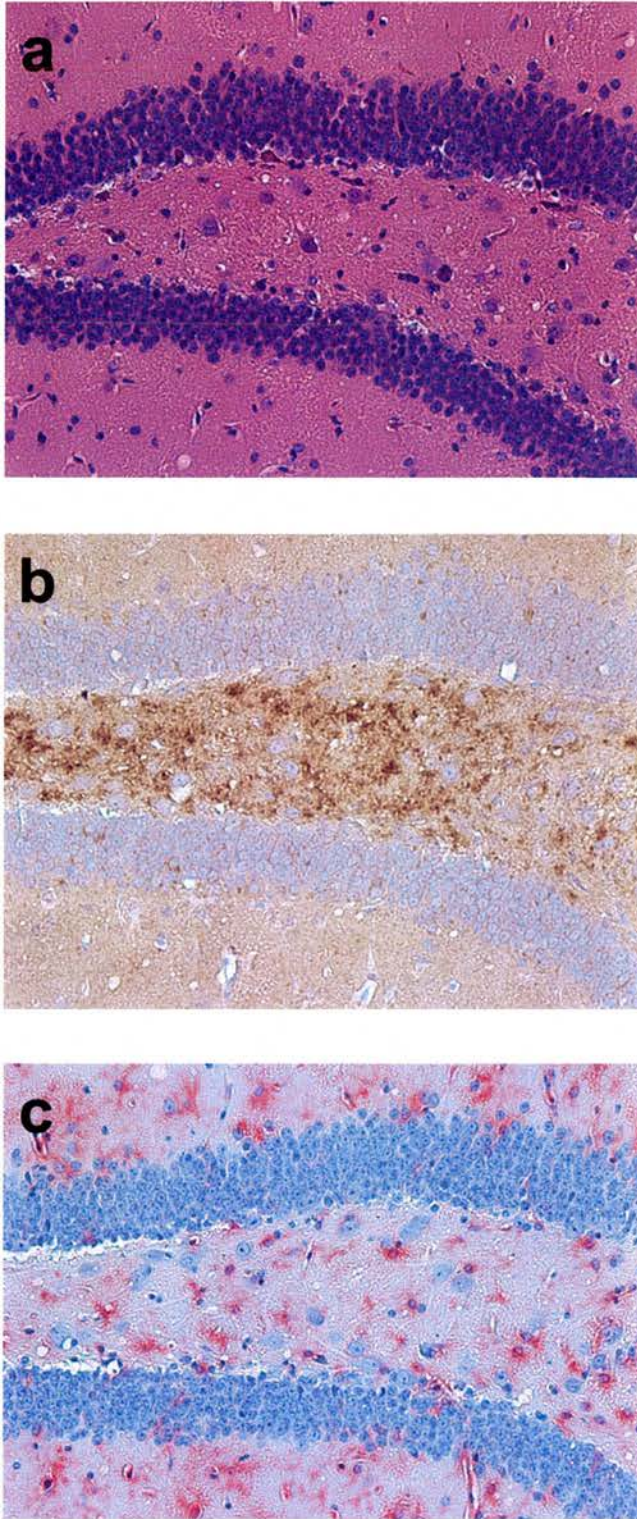
electrophoresis allowing strains to be distinguished (Collinge *et al.*, 1996; Parchi *et al.*, 1995; Somerville, 1999; Somerville *et al.*, 1997b).

TSE disease can be confirmed by the presence of four hallmarks of TSE disease in the brain of an affected individual (Fig 1.2). In a TSE affected brain glial cells will be activated resulting in an increase in size and number of astrocytes (Bruce *et al.*, 1994; Deidrich *et al.*, 1991) and microglial cells (Williams *et al.*, 1997). Loss of neurones will also be evident (Fraser, 2002). These features occur in every case of TSE disease, but may also occur in the brains of individuals affected by other neurological diseases. Characteristic vacuolar pathology (Fraser & Dickinson, 1973) and PrP<sup>Sc</sup> deposition (DeArmond *et al.*, 1985) will also be observed in the brain of a TSE affected individual and are specific to TSE diseases.

## **1.2 TSEs and the immune system**

### **1.2.1 The role of lymphoid tissues**

Although the pathology caused by TSE diseases is largely confined to central nervous system (CNS) tissues, very few natural cases of acquired TSE disease involve transmission directly to the CNS itself. Many natural TSE diseases are thought to be transmitted via the oral route, for example BSE in cattle (Wilesmith *et al.*, 1991), vCJD in humans (Bruce *et al.*, 1997; Hill *et al.*, 1997), scrapie in sheep and goats (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2003; Heggebo *et al.*, 2000; Keulen *et al.*, 1999), CWD in mule deer and elk (Williams & Young, 1980) and TME in mink (Hartsough & Berger, 1965). Other routes include iatrogenic transmission such as blood transfusion (Llewelyn *et al.*, 2004; Peden *et al.*, 2004) or



**Figure 1.2- Classical hallmarks of TSE disease within the brain.** **a)** characteristic vacuolar pathology after staining with haematoxylin and eosin, **b)** deposition of disease specific prion protein ( $\text{PrP}^{\text{d}}$ ) shown using PrP-specific monoclonal antiserum 6H4 (brown) and **c)** activation of glial cells shown using Glial fibrillary acidic protein (GFAP) -specific monoclonal antiserum (red). All sections counterstained with haematoxylin to distinguish cell nuclei. Original magnification X200.



or implantation of infected tissues (Duffy *et al.*, 1974). Thus, study of the peripheral pathogenesis of TSE disease may allow for the development of therapeutic interventions to prevent the spread of the TSE agent to the CNS where invariably fatal, and, at present, pharmacologically irreversible damage is done to the tissues.

Challenge with a TSE agent does not generate a specific immune response as seen following infection with most other infectious agents (McFarlin *et al.*, 1971). There is no discernible humoral immune response as an absence PrP specific antibody generation has been reported (Chandler, 1959; Clarke & Haig, 1966; Marsh *et al.*, 1970; Pattison *et al.*, 1964; Porter *et al.*, 1973). Similarly, genetic deficiency of Fc receptors has no effect on TSE pathogenesis (Klein *et al.*, 2001). There is also no cell mediated immune response to the TSE agent (Kingsbury *et al.*, 1981). The lack of an immune response is not due to the TSE agent itself being immunosuppressive as responses to other antigenic challenges appear to be normal in scrapie infected mice (Clarke, 1968; Gardiner & Marucci, 1969; Garfin *et al.*, 1978). The lack of a TSE specific immune response is likely due to the fact that a major or sole component of the infectious TSE agent is very similar to PrP<sup>Sc</sup>, derived from the host protein PrP<sup>C</sup> which is expressed on many tissues (Manson *et al.*, 1992; Oesch *et al.*, 1985). Thus the host is immunologically tolerant to the TSE agent (Gregoire *et al.*, 2005).

However, there is a role for the immune system in TSE pathogenesis. The early detection of the TSE agent in lymphoid tissues, prior to detection in the CNS or onset of clinical signs of disease in many natural and experimental TSEs suggests that lymphoid tissues do play a role in the peripheral pathogenesis of disease (Andreoletti

*et al.*, 2000; Hadlow *et al.*, 1987; Heggebo *et al.*, 2003; Heggebo *et al.*, 2002; Heggebo *et al.*, 2000; Hilton *et al.*, 1998; Keulen *et al.*, 1996; Keulen *et al.*, 1999; Mabbott *et al.*, 2003; Mohan *et al.*, 2005a; Sigurdson *et al.*, 1999). Early studies showed that splenectomy prior to intra-peritoneal (i.p.) inoculation of the scrapie agent delayed the onset of clinical disease thus demonstrating that the early accumulation of the TSE agent in lymphoid tissues is important for the efficient development of disease, although the spleen is not the only place neuroinvasion occurs from (Clarke & Haig, 1971; Fraser & Dickinson, 1970, , 1978). Splenectomy studies using sub-cutaneous, intra-gastric or intra-ocular routes of inoculation have demonstrated an inconsistent or no role for the spleen, again suggesting that neuroinvasion can take place from other tissues in the absence of the spleen (Fraser *et al.*, 1992; Fraser, 1996; Kimberlin & Walker, 1989). Studies have also shown that the induction of lymphocytic foci by chronic inflammation allows the accumulation of the TSE agent in organs in which this would not otherwise occur (Heikenwalder *et al.*, 2005; Seeger *et al.*, 2005). The contribution of various cells and molecules within lymphoid tissues to the peripheral pathogenesis of TSEs has since been investigated.

### **1.2.2 The role of lymphocytes**

The role of lymphocytes in TSE pathogenesis has been studied by deleting various subsets of lymphocytes and investigating the effect on disease susceptibility. Early studies in mice showed that thymectomy, which causes a general depletion of T-lymphocytes, prior to experimental peripheral infection with scrapie had no effect on the development of disease (Fraser & Dickinson, 1978; McFarlin *et al.*, 1971). Later studies found mice deficient in specific T lymphocyte subsets (e.g. CD4, CD8,

TCR $\alpha$ ) to be as susceptible to scrapie disease as wild-type mice (Klein *et al.*, 1997). A further study has shown that mice with a combined absence of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes at the time of inoculation had delayed onset of disease (Lewicki *et al.*, 2003). However, this effect was found after both peripheral inoculation (i.p.) and direct inoculation of the CNS (intra-cranial inoculation) suggesting that T lymphocytes were enhancing the pathogenesis of disease in the CNS rather than in the periphery. Taken together these data demonstrate T lymphocytes do not contribute to the peripheral pathogenesis of TSEs.

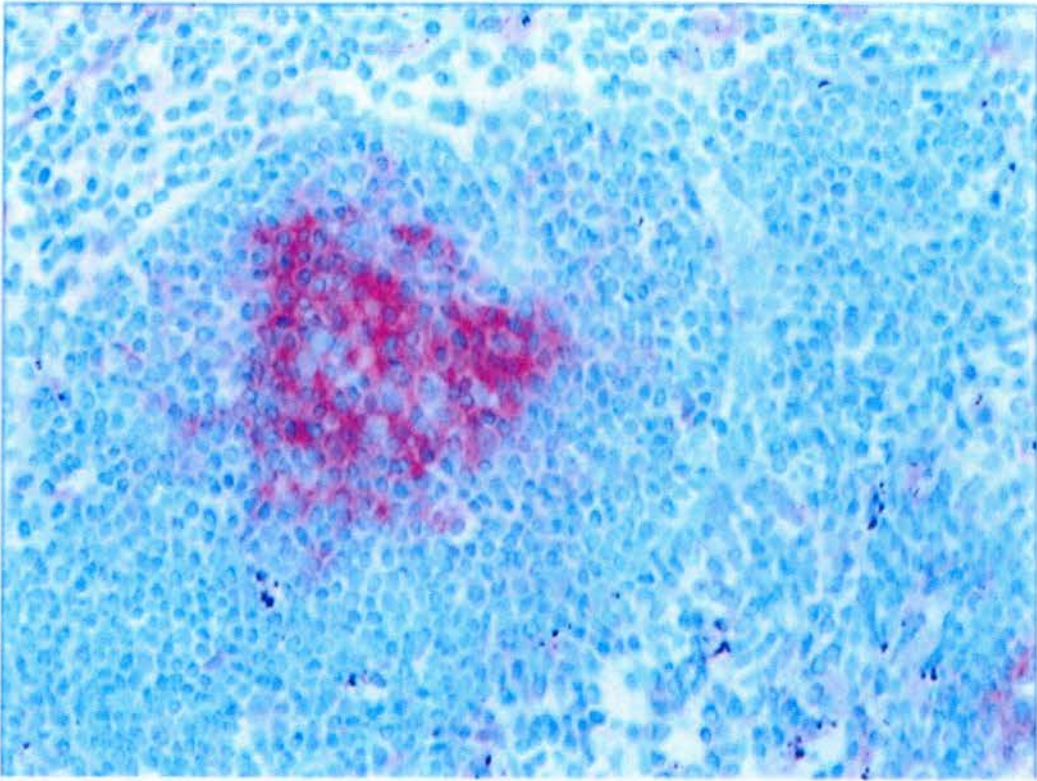
Experiments investigating the role of B lymphocytes, however, showed these cells do play a role in the peripheral pathogenesis of TSEs. In contrast to wild-type mice, mice deficient in B lymphocytes do not accumulate infectivity in the spleen or develop neurological disease (Klein *et al.*, 1997). Severe combined immunodeficiency (SCID) mice that have no B or T lymphocytes are also relatively resistant to peripheral scrapie challenge (Fraser *et al.*, 1996; Lazmesas *et al.*, 1996; Taylor *et al.*, 1996b). B lymphocytes can be reconstituted in SCID mice by grafting with wild-type bone marrow. Using bone marrow grafts from either PrP<sup>+/+</sup> mice or PrP<sup>-/-</sup> mice into SCID mice it has been demonstrated that bone marrow derived lymphocytes need not express PrP<sup>C</sup> for disease to occur (Brown *et al.*, 1999b). The PrP status of the recipient mouse itself, not the graft which provides the B lymphocytes, was important in determining whether disease resulted. This is corroborated by the findings of another study which used a transgenic mouse strain expressing high levels of PrP<sup>C</sup> on B lymphocytes only (Montrasio *et al.*, 2001). These transgenic mice did not replicate scrapie in their spleens. As host cells must



express PrP<sup>C</sup> to replicate TSE agents (Bueler *et al.*, 1993; Manson *et al.*, 1994b; Sakaguchi *et al.*, 1995) these data suggest that a non bone marrow derived host cell expressing PrP<sup>C</sup> is important in the peripheral pathogenesis of TSEs. B lymphocytes give vital signals to many other cell types through the use of cytokines (Chaplin & Fu, 1998; Kosco-Vilbois *et al.*, 1997) therefore the absence of B lymphocytes can result in the absence of other cell types as a consequence. Together these results imply that although B lymphocytes play a role, their effect is possibly indirect. An earlier  $\gamma$ -irradiation study in which a sub-lethal whole body dose was given to mice prior to scrapie infection showed these mice were still susceptible to the disease, even though actively dividing lymphocytes had been temporarily depleted (Fraser & Farquhar, 1987). Taken together these data suggest that a non-bone marrow derived, PrP<sup>C</sup> expressing,  $\gamma$ -irradiation resistant cell that relies on the presence of B lymphocytes is critical for TSE pathogenesis in lymphoid tissues.

### **1.2.3 The role of follicular dendritic cells**

Follicular dendritic cells (FDCs) reside in lymphoid follicles and retain antigen on their surface for long periods of time. FDCs have intimate associations with B lymphocytes and are thought to play a crucial role in the selection of antigen binding B lymphocytes during an immune response and the maintenance of B lymphocyte memory (Berg *et al.*, 1995). These cells have been considered to play a role in the pathogenesis of TSE disease due to the fact that they appear to express high levels of PrP<sup>C</sup> in uninfected mice (Brown *et al.*, 1999b; McBride *et al.*, 1992). Accumulation of high levels of PrP<sup>d</sup> have been observed on FDCs from experimentally infected mice (Fig 1.3; Brown *et al.*, 1999b; Mabbott *et al.*, 2000b; McBride *et al.*, 1992),



**Figure 1.3- Disease specific prion protein (PrP<sup>d</sup>) accumulation on follicular dendritic cells in mouse spleen tissue.** PrP<sup>d</sup> was detected using PrP-specific antiserum 1B3 (red). Section was counterstained with haematoxylin (blue) to distinguish cell nuclei. Original magnification X200.



natural sheep scrapie (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2002; Heggebo *et al.*, 2000; Keulen *et al.*, 1999), experimental CWD (Sigurdson *et al.*, 1999) and patients with vCJD (Hilton *et al.*, 1998). Also, FDCs are mitotically inactive cells and therefore relatively resistant to  $\gamma$ - irradiation (Heinen *et al.*, 1995). FDCs are dependent upon signals from B lymphocytes to induce their maturation and remain in a fully differentiated state (Berg *et al.*, 1995). A recent study has also shown that the onset of susceptibility to peripherally inoculated scrapie in neonatal mice coincides with the development and maturation of FDCs as the mice age (Ierna *et al.*, 2006). These characteristics strengthened the argument for the involvement of FDCs in TSE pathogenesis. However, not all TSE models may depend on FDCs for their pathogenesis. Some studies have shown that in the absence of FDCs disease susceptibility is decreased (Horiuchi *et al.*, 2006; Mabbott *et al.*, 2000a; Mabbott *et al.*, 2002; Mabbott *et al.*, 2000b; Mabbott *et al.*, 2003; Montrasio *et al.*, 2000; Prinz *et al.*, 2003b) others that susceptibility is not affected by the absence of FDCs (Klein *et al.*, 1997; Manuelidis *et al.*, 2000).

FDCs need constant stimulation from B lymphocytes via interactions with cytokines to maintain their fully differentiated state. Tumour necrosis factor - $\alpha$  (TNF $\alpha$ ) is one such cytokine, consequently in the absence of stimulation via TNF $\alpha$  FDCs do not develop (MacKay & Browning, 1998; Matsumoto *et al.*, 1996; Pasparakis *et al.*, 1996). B lymphocytes secrete TNF $\alpha$ , which binds to TNF receptor 1 (TNFR1) on the FDC or its precursor cell, allowing the signal to pass on to the inside of the FDC (Beutler & Huffel, 1994). Disrupting this signalling pathway leads to a lack of terminally differentiated FDCs (MacKay & Browning, 1998; Matsumoto *et al.*, 1996;

Pasparakis *et al.*, 1996) but mature lymphocytes are present providing an opportunity to investigate the potential role specifically of FDCs in TSE pathogenesis. After peripheral inoculation of  $\text{TNF}\alpha^{-/-}$  mice with the ME7 scrapie strain,  $\text{PrP}^{\text{Sc}}$  and infectivity do not accumulate in the spleen and susceptibility is reduced (Mabbott *et al.*, 2000b). The  $\text{TNF}\alpha$  signalling pathway can also be disrupted by treatment with a  $\text{TNFR1}$  homologue. This treatment causes FDCs to de-differentiate temporarily (Mabbott *et al.*, 2002). When mice were treated with this reagent prior to peripheral scrapie inoculation the incubation period increased and scrapie accumulation in the spleen decreased. This would suggest that scrapie replication was unable to take place during this period in the spleen due to the de-differentiation of the FDCs, again suggesting an important role for FDCs in TSE pathogenesis. Another study, however, did not support this role for FDCs (Klein *et al.*, 1997). This study used mice genetically deficient in  $\text{TNFR1}$  ( $\text{TNFR1}^{-/-}$  mice). It would be expected that as these mice lack differentiated FDCs they would not succumb to disease. However, the results showed that after peripheral inoculation with the RML scrapie strain there was no appreciable difference between the  $\text{TNFR1}^{-/-}$  mice and their wild-type counterparts in disease susceptibility. This could be explained by the fact that this study involved the use of a different scrapie strain to that of the first two studies (Mabbott *et al.*, 2002; Mabbott *et al.*, 2000b) which raises the possibility that different strains of scrapie may exhibit different host cell tropisms. It is also possible that, as the latter study used a higher dose of scrapie than the others did, the immune system was bypassed and infection proceeded immediately to the CNS with no replication stage necessary. This theory was also put forward by investigators comparing wild-type bone marrow reconstituted SCID mice with SCID mice to

account for the 33% of SCID mice which did develop disease (Lazmesas *et al.*, 1996). TNF $\alpha$  also has other functions, one of which is the activation of macrophages. A subsequent study (Prinz *et al.*, 2002) put forward the theory that in TNF $\alpha^{-/-}$  or TNFR1 $^{-/-}$  mice the lack of FDCs was perhaps compensated for by replication in dormant macrophages. Strong accumulations of PrP<sup>Sc</sup> were detected in macrophages in lymph nodes from scrapie infected TNFR1 $^{-/-}$  mice (see section 1.2.5).

Lymphotoxin exists in two forms – a secreted homotrimer of lymphotoxin  $\alpha$  (LT $\alpha_3$ ) which is a ligand of TNFR1 and TNFR2, and a membrane bound heterotrimer LT $\alpha_1\beta_2$  which signals through LT $\beta$  receptor (LT $\beta$ R; (Pfeffer, 2003). LT $\alpha_1\beta_2$  is another cytokine required for the terminal differentiation of FDCs and is expressed by B lymphocytes. This protein can bind LT $\beta$ R on FDCs or their precursor cells providing a further signal for FDC differentiation (Endres *et al.*, 1999). In the absence of either LT $\alpha_1\beta_2$  or LT $\beta$ R FDCs do not differentiate (Koni *et al.*, 1997; Matsumoto *et al.*, 1996). If FDCs are important for TSE pathogenesis one would expect that interruptions to the LT $\beta$ R signalling pathway would affect TSE susceptibility in similar ways described above for the TNF $\alpha$  pathway. Studies involving the use of a homologue of the LT $\beta$ R (LT $\beta$ R-Ig) to block the LT $\beta$ R signalling pathway found that this treatment caused the FDCs to de-differentiate temporarily (MacKay & Browning, 1998). Treatment with LT $\beta$ R-Ig prior to peripheral scrapie inoculation blocked the accumulation of PrP<sup>Sc</sup> in the spleen, extended the incubation period (Mabbott *et al.*, 2000a; Mohan *et al.*, 2005a; Montrasio *et al.*, 2000) and reduced disease susceptibility and even prevented the



onset of disease in mice which had no functional FDCs at the time of oral inoculation (Mabbott *et al.*, 2003). Another study (Prinz *et al.*, 2002), using RML scrapie and mice genetically deficient in various components of the LT pathway found that in all cases, resistance was conferred suggesting that replication of scrapie was unable to occur due to the lack of FDCs or other LT-dependent cells caused by disruption of LT signalling. One study, however, using LT $\beta$  knockout mice and a mouse passaged CJD agent found no difference between wild type and knockout mice (Manuelidis *et al.*, 2000) implying no role for FDCs in TSE pathogenesis. This again raises the issue of different strains of TSE exhibiting different host cell tropisms.

#### **1.2.4 Role of complement**

Complement is a series of proteins of the innate immune system that complement (hence the name) the activities antibody. Various characteristic molecular patterns on pathogens, or antibody:antigen complexes can activate the complement cascade. This results in the opsonisation of pathogens, the lysis of infected cells or the induction of an inflammatory response (Walport, 2001). The binding of opsonised antigen to FDCs is thought to enhance the development of immunological memory (Berg *et al.*, 1995; Walport, 2001). It has been proposed that the attachment of the TSE agent to FDCs via interactions with complement is another crucial part of the pathogenesis of TSE diseases (Klein *et al.*, 2001; Mabbott *et al.*, 2001).

One stage of the complement cascade results in C3 being deposited on the surface of pathogens from which one possible outcome is the attachment of the antigen to FDCs through the binding of C3 with complement receptor CR1 (Berg *et al.*, 1995;

Walport, 2001). When there is no C3 present at the time of scrapie agent inoculation, either through genetic deficiency or temporary depletion by treatment with cobra venom factor, accumulation of the scrapie agent in the spleen was reduced and that the incubation period was lengthened (Klein *et al.*, 2001; Mabbott *et al.*, 2001). Mice deficient in the *Cr2* gene which lack the complement receptors CR2/CR1 (CD21/CD35) also show delayed onset of clinical scrapie disease (Klein *et al.*, 2001). As C3d (a C3 breakdown product) is the ligand for CR2/CR1 these data suggest that the attachment of the scrapie agent to FDCs via interactions with complement component C3 and complement receptors CR2/CR1 is important for the development of scrapie disease.

The deposition of C3 on the surface of a pathogen can be mediated by a chain of events beginning with the activation of C1q and involving C2 (classical pathway), or a separate chain of events involving Factor B and the spontaneous hydrolysis of C3 (alternative pathway; Walport, 2001). Studies have also investigated the effect of genetic deficiency of C1q (C1q<sup>-/-</sup> mice) or both factor B and C2 (H2-Bf/C2<sup>-/-</sup> mice) on scrapie pathogenesis. C1q may bind to antibody that has bound antigen and these complexes can adhere to CR1 (Tas *et al.*, 1999) or C1q receptor (Norsworthy *et al.*, 1999) on the FDC directly, thus attaching the antigen. C1q, C2 and Factor B can also bind indirectly to FDCs by activating the complement cascade, which results in the deposition of C3 on the antigen surface, and subsequent attachment to the FDC as described above (Berg *et al.*, 1995; Walport, 2001). Interestingly, C1q has been shown to bind directly to a structurally altered form of PrP (Blanquet-Grossard *et al.*, 2005). An increase in incubation time and reduction in scrapie accumulation in the

spleen was noted when C1q<sup>-/-</sup> mice or H2-Bf/C2<sup>-/-</sup> mice were inoculated with the scrapie agent (Klein *et al.*, 2001; Mabbott *et al.*, 2001). These results suggest that the classical pathway is most likely involved in the attachment of scrapie to FDCs. The effects on scrapie pathogenesis in C1q<sup>-/-</sup> mice are more profound than in mice deficient in complement components downstream of C1q which suggests that the scrapie agent may also be attached directly to FDCs via interactions with C1q. This is further supported by evidence that FDCs themselves can synthesise C1q (Schwaebble *et al.*, 1995).

An alternative explanation of the role of C1q comes from a study which showed that the expression of PrP<sup>C</sup> by FDCs was increased during infection with vesicular stomatitis virus (Lotscher *et al.*, 2003). In C1q<sup>-/-</sup> mice this upregulation did not happen which suggests PrP<sup>C</sup> expression may be regulated by C1q. Thus in the absence of C1q the peripheral pathogenesis of scrapie may be impaired due to lower expression of PrP<sup>C</sup> by FDCs. This also adds weight to the hypothesis that inflammation at the time of TSE inoculation can enhance the pathogenesis of TSEs (Heikenwalder *et al.*, 2005; Seeger *et al.*, 2005).

The deposition of C3b on the surface of a pathogen can lead to the lysis of that pathogen- by the formation of the membrane attack complex (MAC) which consists of complement components C5-C9 (Walport, 2001). There may be a role for complement components and the MAC in neurodegeneration as complement components have been detected in the brains of humans affected by vCJD, fCJD, sCJD and GSS (Ishii *et al.*, 1984; Kovacs *et al.*, 2004) and rodents affected by



scrapie or BSE (Dandoy-Dron *et al.*, 2000; Riemer *et al.*, 2000). However, increased expression of clusterin - a negative regulator of the MAC has also been detected in the brains of natural and experimental cases of TSEs (McHattie *et al.*, 1999; Sasaki *et al.*, 2006) and no differences are observed in the neuropathology induced when mice deficient in various complement components (C1q, C2, C3 and C5) are inoculated with scrapie (Klein *et al.*, 2001; Mabbott & Bruce, 2004; Mabbott *et al.*, 2001) which would suggest that whatever the effect of the activation of complement is in a TSE affected brain, neurodegeneration itself is not mediated by complement.

#### **1.2.5 The role of macrophages**

Another cell type that resides in the germinal centres of lymphoid tissues and is possibly radioresistant is the tingible body macrophage. These cells play a role in the destruction of foreign protein and host cell debris (Smith *et al.*, 1991; Swartzendruber & Congdon, 1963) so it is possible that they will take up the TSE agent as part of their normal activities. Studies have shown the accumulation of the scrapie agent in macrophages in spleens of scrapie infected mice (Jeffrey *et al.*, 2000), Peyer's patches (PPs) of scrapie infected hamsters (Beekes & McBride, 2000), lymph nodes of scrapie infected sheep (Herrmann *et al.*, 2003) and tonsils of CWD infected mule deer (Sigurdson *et al.*, 2002). Also, as mentioned earlier, it has been suggested that accumulation or replication of scrapie might occur in macrophages that are not activated – for example, in lymph nodes of TNFR1<sup>-/-</sup> (Prinz *et al.*, 2002). This study found that PrP colocalised with markers for macrophages in mice lacking functional genes for molecules of the TNFR1 signalling pathway.

However, the significance of this accumulation is not clear. A body of evidence points to macrophages playing a preventative role in the peripheral pathogenesis of TSEs. It is possible to deplete macrophages by treatment with clodronate (Beringue *et al.*, 2000). When such experiments were carried out it was found that far from preventing disease as would be expected if macrophages were aiding scrapie pathogenesis, this treatment actually enhanced pathogenesis thereby suggesting that macrophages in the spleen play a preventative role in a natural situation (Beringue *et al.*, 2000; Maignen *et al.*, 2005). This idea is consistent with data from *in vitro* studies which found that scrapie infectivity decreased over time when incubated with macrophages (Carp & Callaghan, 1981, , 1982). However, clodronate also transiently depletes FDCs and B lymphocytes in the spleen thus the effect of clodronate treatment on scrapie pathogenesis could be attributed to the absence of other cell types. Further *in vivo* work is clearly required to ascertain the precise role of macrophages in TSE pathogenesis.

#### **1.2.6 Transport of the TSE agent to the lymphoid tissue from the site of inoculation**

From the work discussed so far it is possible to conclude that lymphoid tissues and various components of the immune system are involved in the peripheral pathogenesis TSE disease. The way in which the TSE agent reaches the lymphoid tissues from the site of inoculation is not clear but may involve dendritic cells (DCs), M cells (when infection occurs via the gastro-intestinal tract) or a cell free pathway. DCs have a completely different origin and function so should not be confused with FDCs mentioned earlier. DCs are bone marrow derived and function to sample their

local environment in the periphery, capture antigen, migrate to lymphoid organs and present the antigen to T lymphocytes to initiate an immune response against the antigen (Banchereau & Steinman, 1998). It is quite plausible, therefore, that DCs might take up the TSE agent at the point of entry in the course of their natural activity. *In vitro* work has shown immature DCs respond chemotactically to a fragment of PrP (Kaneider *et al.*, 2003) and can acquire and retain TSE agents in native form (Huang & MacPherson, 2004; Mohan *et al.*, 2005c). These studies suggest DCs would make ideal candidates for the transport of the TSE agent from the site of inoculation. However, DCs do not generally migrate to the B lymphocyte follicles of lymphoid tissues where FDCs are found (Shortman & Lui, 2002), although there is a subset of DCs which do (Berney *et al.*, 1999; Yu *et al.*, 2002). *In vivo* work has shown that in lymph draining from the intestine, PrP<sup>d</sup> was associated with a small percentage of DCs, but not present in other cells (Huang *et al.*, 2002). These PrP<sup>d</sup>-positive DCs were found not to be infectious, however, this could be due to the numbers of PrP<sup>d</sup>- positive cells being too small to transmit disease in a bioassay. PrP<sup>Sc</sup> was not detected in any DC population after cannulation of the draining afferent lymphatics of the site of inoculation in an *in vivo* study in sheep (Gossner *et al.*, 2006) suggesting that DCs have no role in the transport of the agent. The migration of Langerhans cells from the skin to the draining lymph node is impaired in CD40L<sup>-/-</sup> mice (Moodycliffe *et al.*, 2000). When these mice were challenged with scrapie via scarification of the skin of the thigh, disease progression was not impaired suggesting that Langerhans cells are not important for transport of the scrapie agent from the site of inoculation (Mohan *et al.*, 2005b). However, this does not exclude a role for other populations of migratory DCs in the skin such as



dermal dendritic cells (Lenz *et al.*, 1993) which can provide a Langerhans cell-independent pathway of presentation of antigen from the skin (Kurimoto *et al.*, 1994; Streilein, 1989), or Langerhans cells migrating under steady state conditions rather than cells actively stimulated to migrate (Yoshino *et al.*, 2003). DCs have been shown to open tight junctions in the intestinal epithelium and sample the contents of the intestinal lumen (Rescigno *et al.*, 2001), suggesting that DCs could be involved in the direct uptake of the TSE agent from the intestine. DCs remain, therefore, a prime candidate for the transport of the TSE agent from the site of inoculation to the local lymphoid tissues but further studies are required to confirm this. DCs may also play a role in protection against TSE diseases as studies have shown that they can degrade the TSE agent over time (Mohan *et al.*, 2005c; Rybner-Barnier *et al.*, 2006).

Many natural TSEs are likely to be acquired via the oral route. M-cells are specialised cells in the intestinal epithelium which sample antigens from the intestinal lumen and translocate them across the epithelium for uptake by antigen presenting cells such as DCs (Neutra *et al.*, 1996). Using an *in vitro* M-cell model it has been shown that M-cells are capable of taking up the scrapie agent which suggests they may transfer TSE agents across the intestinal epithelium (Heppner *et al.*, 2001), although *in vivo* confirmation of this is required. Stomach enzymes may partially degrade PrP<sup>d</sup> to its 27-30 kDa PK resistant core (Jeffrey *et al.*, 2006; Mishra *et al.*, 2004). *In vitro* evidence suggests it is possible that these fragments may be able to cross the intestinal epithelium through being taken up by enterocytes (Mishra *et al.*, 2004; Morel *et al.*, 2005). This idea is supported by studies showing that the laminin receptor present on enterocytes can act as a receptor for PrP<sup>Sc</sup> (Gauczynski *et*

*al.*, 2006; Morel *et al.*, 2005). The transit of the TSE agent across an intestinal epithelial model also appears to be dependent on ferritin, an intriguing finding as ferritin is found in high quantities in meat (Mishra *et al.*, 2004).

### **1.2.7 Transport of the TSE agent from the lymphoid tissue to the CNS**

The spread of TSE agents from the sites of replication in lymphoid tissue to the CNS is thought to occur via the peripheral nervous system. Early investigations in mice found that after peripheral challenge replication of scrapie in the CNS consistently began in the thoracic region of the spinal cord with replication in the brain and other parts of the spinal cord occurring significantly later (Kimberlin & Walker, 1980). This suggested that neuronal rather than haematological spread was occurring as this ordered sequence of events would not be expected if the agent was spreading via the bloodstream. In experimentally infected rodents PrP<sup>d</sup> has been found in peripheral nerves prior to detection in the CNS (Baldauf *et al.*, 1997; Beekes *et al.*, 1996; Beekes & McBride, 2000; Beekes *et al.*, 1998; McBride & Beekes, 1999; McBride *et al.*, 2001). The involvement of the enteric nervous system in natural TSE disease has been shown by the accumulation of PrP<sup>d</sup> in these nerves in cases sheep scrapie (Heggebo *et al.*, 2003), humans with vCJD (Haik *et al.*, 2003) and deer affected by CWD (Sigurdson *et al.*, 2001). Further evidence of the involvement of peripheral nerves has come from a study showing that FDCs abnormally close to the nerves in the spleen of CXCR5<sup>-/-</sup> mice speeds up disease progression (Prinz *et al.*, 2003a).

### **1.3 Development of secondary lymphoid tissue**

From the work discussed so far it can be seen that although knowledge of the peripheral pathogenesis of TSEs is accumulating many questions remain unanswered. One such question is; from which lymphoid tissues does neuroinvasion occur? Does neuroinvasion occur solely from the lymphoid tissues draining the site of inoculation in which the initial accumulation of the TSE agent has been observed (Andreoletti *et al.*, 2000; Hadlow *et al.*, 1987; Heggebo *et al.*, 2003; Heggebo *et al.*, 2002; Heggebo *et al.*, 2000; Hilton *et al.*, 1998; Keulen *et al.*, 1996; Keulen *et al.*, 1999; Mabbott *et al.*, 2003; Mohan *et al.*, 2005a; Sigurdson *et al.*, 1999) or does neuroinvasion occur from many tissues such that the absence of the draining lymphoid tissue can be compensated for by others? These issues are addressed in this thesis. The approach taken here is to utilise mice that lack various lymphoid tissues due to the disruption of signals involved in the organogenesis of lymphoid tissues.

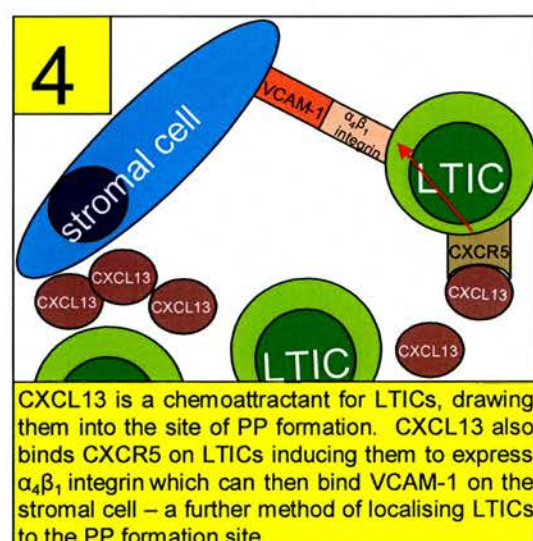
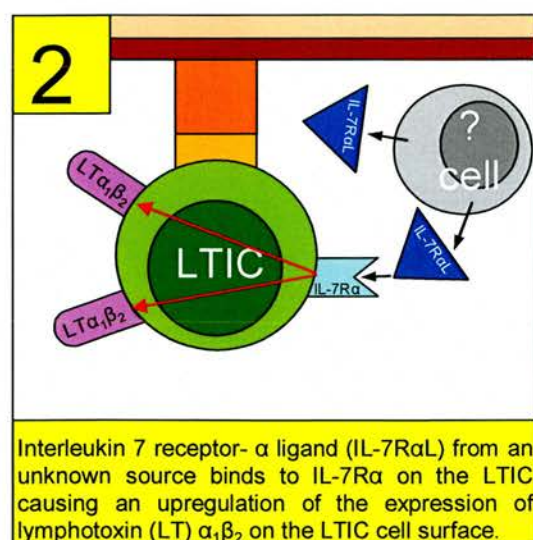
In mice, the development of secondary lymphoid organs does not occur by a uniform process – different lymphoid organs develop using different molecular pathways and occur at different times during gestation. For example, experiments using LT $\beta$ R-Ig to block LT $\beta$ R signalling during embryonic development showed that treatment at distinct times blocked the formation of distinct lymphoid organs (Rennert *et al.*, 1996). The formation of the spleen was not affected by this treatment although the microarchitecture was altered. This allows for the creation of mice that lack specific lymphoid tissues (e.g. Peyer's patches and lymph nodes) but retain the spleen.



### **1.3.1 Development of Peyer's patches**

The first time it is possible to visualise the formation of the Peyer's patches (PPs) in the mouse is on the antimesenteric side of the intestine at about 15 days of gestation. At this stage the early PP appears as a cluster of vascular cell adhesion molecule (VCAM) –1 and intercellular adhesion molecule (ICAM) –1 expressing stromal cells of mesenchymal origin and lymphoid tissue initiating cells (LTICs) of lymphoid origin that are  $CD4^+CD3^-$  and express IL7R $\alpha$  and  $\alpha_4\beta_7$  integrin (Adachi *et al.*, 1997; Finke *et al.*, 2002; Mebius *et al.*, 1996). This cluster is known as the PP anlage (Fig 1.4, Step 5). The development of LTICs is thought to be dependent on the transcription factors, Ikaros (Georgopoulos *et al.*, 1994; Wang *et al.*, 1996) and Id2 (Yokota *et al.*, 1999) and the nuclear hormone receptor retinoid-related orphan receptor- $\gamma$  (Kurebayashi *et al.*, 2000; Sun *et al.*, 2000). In mice genetically deficient in these molecules lymph nodes (LNs) and PPs do not develop and LTICs are not detected in the rudimentary lymphoid tissue anlage. This also demonstrates that the formation of PPs and all LNs is crucially dependent on LTICs.

The first interaction for the formation of PP anlagen may be between  $\alpha_4\beta_7$  integrin on the LTIC and mucosal addressin cell adhesion molecule (MAdCAM) –1 on venous endothelial cells overlying the foetal intestine (Fig 1.4, Step 1). MAdCAM-1 has been found to be expressed on these endothelial cells from day 13.5 of gestation (Hashi *et al.*, 2001) and LTICs are known to express  $\alpha_4\beta_7$  integrin (Mebius *et al.*, 1996), but it is not clear whether this interaction does occur as an initiation step, or





later on. In any case this interaction is not vital as experiments that blocked this interaction from 10 days gestation failed to completely prevent the accumulation of LTICs (Mebius *et al.*, 1996) and normal numbers of PPs are found in  $\beta_7$  integrin<sup>-/-</sup> mice (Wagner *et al.*, 1996).

What is certain is a ligand that binds to IL-7R $\alpha$  (IL-7R $\alpha$ L) from an unknown source binding to IL-7R $\alpha$  on LTICs is a vital step in the formation of the PP anlage as mice lacking the receptor have no PPs (Adachi *et al.*, 1998; Yoshida *et al.*, 1999). This ligand is not IL-7 itself since IL-7<sup>-/-</sup> mice were found to have PPs (Nishikawa *et al.*, 2003). This is in contrast to other studies that did not detect the macroscopic development of PPs in IL-7<sup>-/-</sup> mice (Freedden-Jeffrey *et al.*, 1995; Laky *et al.*, 2000) macroscopically. However, it can be argued that, as IL-7<sup>-/-</sup> mice are also severely lymphopenic, macroscopic analysis would not be sensitive enough to find PP structures when few lymphocytes are present. IL-7R $\alpha$  combines with IL-2R $\gamma$  ( $\gamma_c$ ) to form the IL-7R (Kondo *et al.*, 1994), which implies that IL-7 cannot bind to IL-7R $\alpha$  itself and is, therefore, unlikely to be the ligand which associates with IL-7R $\alpha$  during the formation of PP anlagen. The ligand that binds IL-7R $\alpha$  during the generation of PP anlagen is also unlikely to be the other known IL-7R $\alpha$  ligand, thymic stromal lymphopoietin (TSLP), as the signal transduction pathway for TSLP has been very well characterised and does not involve the signalling molecules that induce the expression of molecules involved in PP anlage formation (Park *et al.*, 2000). This implies there is a third, as yet unidentified, ligand for IL-7R $\alpha$  that is employed in the formation of PPs. It was found that blocking the action of this unknown ligand using an antibody to IL-7R $\alpha$  prevented the expression of LT $\alpha_1\beta_2$  by the LTICs. *In vitro*



LTICs could be induced to express  $LT\alpha_1\beta_2$  using rIL-7 - an expression that could also be suppressed by preincubation with the same antibody mentioned above (Honda *et al.*, 2001). This suggests that the role of IL-7R $\alpha$ L in PP formation is to induce the expression of  $LT\alpha_1\beta_2$  in LTICs (Fig 1.4, Step 2).

It is well documented that mice lacking either  $LT\alpha$  or  $LT\beta$  or treated with an agent to block the interaction of  $LT\alpha_1\beta_2$  with its receptor  $LT\beta$ R completely lack PPs (Banks *et al.*, 1995; Honda *et al.*, 2001; Koni *et al.*, 1997; Rennert *et al.*, 1996; Togni *et al.*, 1994; Yoshida *et al.*, 1999), thus demonstrating a vital role for these molecules. *In utero* treatment with a  $LT\beta$ R agonist in  $LT\alpha^{-/-}$  mice results in the formation of lymph nodes in the progeny, further demonstrating the importance of  $LT\beta$ R signalling for the formation of lymphoid tissues (Rennert *et al.*, 1998). It has also been shown that stromal cells express  $LT\beta$ R (Honda *et al.*, 2001), the receptor for the  $LT\alpha_1\beta_2$  heterotrimer. Thus LTIC-derived  $LT\alpha_1\beta_2$  interacts with  $LT\beta$ R expressed by the stromal cells, to induce important downstream effects. Signalling through  $LT\beta$ R is known to trigger two separate nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathways of signal transduction. The first involves the activation of inhibitor of  $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) and the subsequent formation of p50:RelA heterodimer – a transcription factor involved in the transcription of VCAM-1 and the protein p100 (Dejardin *et al.*, 2002). The second involves the activation of NF- $\kappa$ B inducing kinase (NIK) that activates the inhibitor of  $\kappa$ B kinase- $\alpha$  (IKK $\alpha$ ). Together NIK and IKK $\alpha$  degrade p100 to p52 that forms a heterodimer with RelB to become a transcription factor involved in the transcription of chemokines including CXCL13 (Dejardin *et al.*, 2002; Yilmaz *et al.*, 2003). It is known that stromal cells express VCAM-1 on their surface and secrete

chemokine CXCL13, both of which are critical for PP formation, in a IL-7R $\alpha$ -dependent manner (Honda *et al.*, 2001). Together these data strongly suggest that the role of LT $\alpha_1\beta_2$  in PP formation is to induce the expression of VCAM-1 and CXCL13 by the stromal cells (Fig 1.4, Step 3).

The next step is to determine the role these molecules may play in the formation of PPs. CXCL13 (also known as B lymphocyte chemoattractant, BLC) plays a role in the homing and positioning of B cells in lymphoid tissue in an adult. In an embryo it appears to act strongly as a chemoattractant for LTICs (Honda *et al.*, 2001). LTICs have also been found to express the receptor (CXCR5) for CXCL13. Signalling through this receptor induces the activation of  $\alpha_4\beta_1$  integrin on the LTIC which then allows the binding of  $\alpha_4\beta_1$  integrin to the VCAM-1 on the stromal cell (Fig 1.4, Step 4). The interaction of  $\alpha_4\beta_1$  integrin and VCAM-1 has been found to be essential for the clustering of LTICs and stromal cells (mentioned above as the first visible sign of PP formation; Fig 1.4, Step 5; Finke *et al.*, 2002).

These data fit together to present a coherent pathway for the formation of PP anlagen (Fig 1.4). However, there appear to be some conflicts as to whether the required molecules are actually expressed at the necessary times according to the proposed pathway. For example, it is commonly agreed that the first appearance of the PP anlagen is at about 15.5 days of gestation, which would imply that the pathway discussed above takes place before this time. This is supported by IL-7R $\alpha$  being found to be critical from about 14 days gestation (Yoshida *et al.*, 1999) in experiments using IL-7R $\alpha$ - specific antibody injections at various times. However,



immunostaining experiments could not detect IL-7R $\alpha$  expression before day 15.5 (Adachi *et al.*, 1997), and also CXCL13 was not detectable before day 17.5 (Honda *et al.*, 2001). This may be due, however, to the level of sensitivity of the immunostaining being insufficient to detect the output of just a few cells in the early stages of PP anlage formation. There also appears to be conflict as to the role of TNF $\alpha$  and TNFR1 in the formation of PPs. Some studies show a reduced number of PPs in either TNF $\alpha$ <sup>-/-</sup> (Korner *et al.*, 1997; Pasparakis *et al.*, 1997) or TNFR1<sup>-/-</sup> (Pasparakis *et al.*, 1997) and some studies showing no PPs at all in these mice (Kuprash *et al.*, 2005; Neumann *et al.*, 1996). The development of PPs appears to be affected to some extent by the absence of either TNF $\alpha$  or TNFR1 in all the studies mentioned, suggesting that TNF $\alpha$  and TNFR1 do play a role in PP development although precisely what that role is remains to be determined. There does not appear to be any evidence as to whether LTICs and/or stromal cells in the PP anlagen express either of these molecules.

We now need to consider how the homogeneous PP anlage forms into the highly organised PP — a process termed compartmentalisation. Whole mount immunostaining has shown the migration of LTICs to the edge of the PP anlage to form the centre of the follicle beginning on day 18 gestation and finishing about day 2 after birth (Hashi *et al.*, 2001). This study also showed that, in the same time frame, the stromal cells also migrate and are arranged around the outside of the clusters of LTICs. An influx of mature lymphocytes was also noted around gestational day 18, and it was initially thought that this could be the trigger for the migration of the anlage cells. This proved not to be the case as SCID mice that lack



mature lymphocytes were found to show compartmentalisation of PP anlagen (Hashi *et al.*, 2001). The molecular mechanisms that are involved in compartmentalisation are not well understood.

Cells with the same phenotype as LTICs ( $CD3^-CD4^+$ ) and similar gene expression profiles have been found in the spleen and lymph nodes of adult mice (Kim *et al.*, 2003; Kim *et al.*, 2006). These cells are found within the B lymphocyte follicles and at the interface between the follicles and adjoining T lymphocyte areas (Kim *et al.*, 2003). They also express high levels of T lymphocyte co-stimulatory molecules OX40 ligand (OX40L) and CD30 ligand (CD30L; Kim *et al.*, 2005; Kim *et al.*, 2006). This has led to the suggestion that LTICs in adult lymphoid tissues play a role in supporting T lymphocyte help for B lymphocytes both during affinity maturation and the induction of memory antibody responses (Gaspal *et al.*, 2005). Thus, having been involved in the development of an organ, LTICs then go on to support one of the crucial functions of that organ.

### **1.3.2 Development of peripheral lymph nodes**

Although the general principles behind the formation of all lymphoid tissues are the same (generation of anlagen by the interaction of LTICs with stromal cells, then compartmentalisation and influx of mature lymphoid cells) the molecular mechanisms bringing about these events are slightly different for different types of lymphoid tissues.

The first event in the development of lymph nodes is the budding of endothelial cells from major veins to form lymph sacs. Connective tissue protrudes into these sacs, pushing them outwards, eventually allowing the sacs to form the subcapsular sinus of the lymph node. Lymph vessels sprout from the lymph sacs into the surrounding tissues where they eventually meet and attach to lymph vessels sprouting from other lymph node anlagen, thus leading to the development of the complete lymphatic vasculature (Wigle & Oliver, 1999). The main difference between PP and lymph node (LN) formation is that there is another essential molecule, TNF-related activation induced cytokine (TRANCE, also known as RANKL, OPGL, ODF or TNFSF11). Mice deficient in TRANCE (TRANCE<sup>-/-</sup> mice) do not develop lymph nodes, although the PPs are intact (Kim *et al.*, 2000). TRANCE<sup>-/-</sup> mice showed decreased numbers of LTICs and no LN anlage formation although some LTICs were still present suggesting that TRANCE is not involved in their differentiation. This defect could be restored by transgenic TRANCE expression in TRANCE<sup>-/-</sup> mice but not in LTα<sup>-/-</sup> mice suggesting LTα<sub>1</sub>β<sub>2</sub> signalling is required in addition to TRANCE signalling (Kim *et al.*, 2000). TRANCE has been shown to induce LTα<sub>1</sub>β<sub>2</sub> expression by LTICs (Yoshida *et al.*, 2002). Bypassing this interaction by the injection of a LTβR agonist into TRANCE<sup>-/-</sup> embryos did not result in the formation of LNs (Kim *et al.*, 2000), however, suggesting that TRANCE plays additional vital roles in LN development. The signalling pathway utilised by TRANCE for the formation of lymph nodes likely involves both TRANCE receptor (TRANCER) and TNF receptor associated factor 6 (TRAF6) as both TRANCER<sup>-/-</sup> mice and TRAF6<sup>-/-</sup> mice also lack all lymph nodes (Dougall *et al.*, 1999; Naito *et al.*, 1999).

### **1.3.3 Development of mesenteric lymph nodes**

There is a striking contrast in the formation of mesenteric lymph nodes (MLNs) as compared to other LNs and PPs in that they do not depend on  $LT\alpha_1\beta_2$  signalling, the central interaction in the formation of those lymphoid tissues mentioned so far. Mice lacking  $LT\beta$  have MLNs (Koni *et al.*, 1997) and those lacking  $LT\alpha$  have MLNs only 2-4% of cases where one single MLN is found (Banks *et al.*, 1995; Togni *et al.*, 1994). Experiments have been carried out to determine if preventing the expression of another gene in conjunction with  $LT\beta$  can prevent the formation of MLNs. Lack of TNF receptor (TNFR1; Koni & Flavell, 1998; Rennert *et al.*, 1998) and  $LT\beta R$  (Futterer *et al.*, 1998) did block the formation of MLNs, whereas knocking out the TNF gene did not (Kuprash *et al.*, 1999). This suggests that the formation of MLNs is reliant on signalling through these receptors triggered by an as yet unidentified ligand. The absence of MLNs from  $LT\alpha^{-/-}$  mice (Banks *et al.*, 1995; Fu *et al.*, 1997) and  $LT\beta R^{-/-}$  mice (Futterer *et al.*, 1998) and comparison of the levels of  $LT\beta R$  expressed by the cells present in MLN anlage compared to other lymph node anlage (Cupedo *et al.*, 2004b) gives some indication that in the absence of  $LT\alpha_1\beta_2$ , MLNs may form by the interaction of  $LT\alpha_3$  with  $LT\beta R$ . However, there is no published evidence to suggest that  $LT\alpha_3$  is a ligand for  $LT\beta R$ . The formation of MLNs is also independent of CXCR5 and CXCL13 as MLNs develop in mice deficient in either of these molecules (Ansel *et al.*, 2000) further demonstrating that different molecules are involved in the formation of MLNs compared to the formation of other lymphoid tissues.



#### **1.3.4 Development of nasal associated lymphoid tissue**

Nasal associated lymphoid tissue (NALT) is very similar to PPs in its structure and function and it was initially thought that the molecular mechanisms involved in its development would be very similar. This, however, was not the case. Immunostaining revealed that NALT was present in  $LT\alpha^{-/-}$ ,  $LT\beta^{-/-}$ ,  $IL-7R^{-/-}$ ,  $TRANCE^{-/-}$ ,  $TNF^{-/-}$  and  $TNFR1^{-/-}$  mice (Fukuyama *et al.*, 2002; Fukuyama *et al.*, 2006; Harmsen *et al.*, 2002), implying that totally different pathways are utilised for the formation of NALT anlagen. NALT forms after birth unlike most other lymphoid tissues (Fukuyama *et al.*, 2002; Harmsen *et al.*, 2002). LTICs were found to be present in NALT anlagen (Fukuyama *et al.*, 2002) suggesting that these cells play a role in the genesis of NALT. NALT does not form in  $Id2^{-/-}$  mice, suggesting the involvement of Id2 in this process, however other ligands and receptors that the NALT forming LTICs express are unidentified at present. As in the spleen, the organisation of the microarchitecture of NALT is disrupted in  $LT\alpha^{-/-}$  mice (Harmsen *et al.*, 2002), but the tissue is present, showing that although different signalling molecules are utilised for the formation of lymphoid tissues, the organisation of lymphoid tissues follows the same pathway.

#### **1.3.5 Development of isolated lymphoid follicles**

As well as PPs, the lymphoid tissue of the murine intestine also includes isolated lymphoid follicles (ILFs; Hamada *et al.*, 2002). The term ILF incorporates a plethora of structures ranging from a loose cluster of B lymphocytes at the base of a villus to highly organised structures the width of one or more villi. ILFs are small solitary follicles unlike PPs that are an aggregation of several large follicles (Lorenz *et al.*,

2003). Like NALT, ILFs are formed postnatally (Hamada *et al.*, 2002). However, unlike NALT, ILF formation is dependent upon the  $LT\alpha_1\beta_2$  -signalling pathway as mice treated continuously with  $LT\beta R$ -Ig,  $LT\alpha^{-/-}$  mice or  $LT\beta R^{-/-}$  mice do not possess them (Lorenz *et al.*, 2003). LTICs appear not to be involved in the organogenesis of ILFs as the source of  $LT\alpha_1\beta_2$  required for their formation has been identified as B lymphocytes, T lymphocytes and NK cells (Lorenz *et al.*, 2003; McDonald *et al.*, 2005).

## **1.4 Aims**

The main aim of this thesis is to investigate the importance of draining lymphoid tissues in TSE agent neuroinvasion. Previous work has shown that the TSE agent accumulates in draining lymphoid tissues prior to spread to non-draining lymphoid tissues or the CNS (Andreoletti *et al.*, 2000; Hadlow *et al.*, 1987; Heggebo *et al.*, 2003; Heggebo *et al.*, 2002; Heggebo *et al.*, 2000; Hilton *et al.*, 1998; Keulen *et al.*, 1996; Keulen *et al.*, 1999; Mabbott *et al.*, 2003; Mohan *et al.*, 2005a; Sigurdson *et al.*, 1999). However, the importance of this stage in the pathogenesis of TSE diseases is not clear. Understanding the importance of this stage may aid the design of therapeutic strategies for the treatment of TSE diseases. In this thesis mice lacking the draining lymphoid tissues for various routes of inoculation were used to assess the importance of particular lymphoid tissues in the pathogenesis of scrapie.

Investigations into the development of secondary lymphoid tissues revealed that  $LT\alpha_1\beta_2$  signalling through  $LT\beta R$  is crucial for the development of most secondary lymphoid tissues. Thus  $LT\alpha^{-/-}$  mice have only spleen and NALT (Banks *et al.*, 1995;

Fukuyama *et al.*, 2002; Togni *et al.*, 1994) and  $LT\beta^{-/-}$  mice have spleen, NALT, cervical lymph nodes (CLNs) and mesenteric lymph nodes (MLNs; Fukuyama *et al.*, 2002; Koni *et al.*, 1997). Mice lacking  $LT\alpha$  or  $LT\beta$  also lack FDCs (Koni *et al.*, 1997; Matsumoto *et al.*, 1996). As FDCs are important for efficient TSE neuroinvasion (section 1.2.3), it was vital to reconstitute these mice with wild-type bone marrow to restore the differentiation of FDCs without inducing the formation of the missing lymphoid tissues (Koni & Flavell, 1998; Mariathasan *et al.*, 1995). This ensured that the results could be attributed to the lack of lymphoid tissues and not the lack of FDCs. Progeny of mice treated with  $LT\beta$ R-Ig during gestation to disrupt LT signalling *in utero* have spleen, NALT, CLNs, MLNs and also lumbar and sacral lymph nodes (Rennert *et al.*, 1997; Rennert *et al.*, 1996). In this instance, LT signalling was temporarily blockaded during gestation and was restored following catabolism of  $LT\beta$ R-Ig promoting subsequent FDC maturation in the progeny mice.

$LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and mice treated *in utero* with  $LT\beta$ R-Ig were inoculated with scrapie to investigate the importance of draining lymphoid tissues in scrapie agent neuroinvasion via the following routes:

1. Oral inoculation. The lymphoid tissues draining this route are the tissues that make up the gut-associated lymphoid tissues (GALT) such as PPs, MLNs and ILFs.  $LT\alpha^{-/-}$  mice lack all GALT,  $LT\beta^{-/-}$  mice and *in utero*  $LT\beta$ R-Ig treated mice lack PPs but possess MLNs. The remaining lymphoid tissues in individual models varies allowing the contributions of non-draining lymphoid tissues to be investigated.



2. Inoculation via scarification of the skin of the thigh. The inguinal lymph nodes are the draining lymphoid tissues of this route.  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and *in utero*  $LT\beta$ R-Ig treated mice lack the inguinal lymph nodes but, again, the specific sets of lymphoid tissues that remain in these models vary allowing the contributions of non-draining lymphoid tissues to be investigated.

3. Inoculation via intra-peritoneal inoculation. The cranial mediastinal lymph nodes drain this route.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack these lymph nodes but, again, the remaining lymphoid tissues in individual models varies allowing the contributions of non-draining lymphoid tissues to be investigated.

Data generated from this thesis permitted an additional point to be addressed: characterisation of isolated lymphoid follicles. These lymphoid tissues have only recently been discovered in the murine small intestine (Hamada *et al.*, 2002; Lorenz *et al.*, 2003). Their cellular characteristics and potential involvement in TSE pathogenesis after oral inoculation was investigated.

# 2

## Materials and Methods

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## **2. Materials and Methods**

### **2.1. Mice**

#### **2.1.1 C57BL/Dk**

This mouse strain has been extensively used at the Neuropathogenesis Unit (NPU), Institute for Animal Health, Edinburgh, in scrapie agent transmission studies.

#### **2.1.2 LT $\alpha$ <sup>-/-</sup>**

LT $\alpha$ <sup>-/-</sup> mice were obtained from B & K Universal Ltd (Hull, UK). Disruption of the *Lta* gene was achieved by insertion of a neomycin resistance cassette in the sense orientation in exon 2 (Riminton *et al.*, 1998). Mice were maintained on a C57BL/6 background. Age matched C57BL/6 mice were used as immunocompetent controls in all experiments using LT $\alpha$ <sup>-/-</sup> mice.

#### **2.1.3 LT $\beta$ <sup>-/-</sup>**

LT $\beta$ <sup>-/-</sup> mice were obtained from B & K Universal Ltd (Hull, UK). Disruption of the *Ltb* gene was achieved by insertion of a neomycin resistance cassette in reverse orientation in exon I (Ngo *et al.*, 1999). Mice were maintained on a C57BL/6 background. C57BL/6 mice were used as immunocompetent controls in all experiments using LT $\beta$ <sup>-/-</sup> mice.

#### **2.1.4 Severe Combined Immunodeficiency (SCID) /Prnp<sup>+/+</sup>**

SCID/Prnp<sup>+/+</sup> mice were produced at NPU by crossing C.B-17 SCID mice with 129/Ola Prnp<sup>+/+</sup> mice (Brown *et al.*, 1999b; Manson *et al.*, 1994a). At the F2 generation mice were selected for the SCID phenotype and Prnp<sup>+/+</sup> genotype. These

mice were used as negative controls for the measurement of faecal IgA by ELISA (section 2.4.5).

### **2.1.5 Housing**

All mice used in these studies were housed under specific pathogen free conditions in a conventional animal facility with a 12 h light and 12 h dark cycle. Immunodeficient mice (SCID mice,  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and C57BL/Dk mice treated *in utero* with  $LT\beta$ R-Ig) were housed in individually ventilated cages. All experiments were conducted under the provisions of the Animals (Scientific Procedures) Act 1986.

## **2.2 Pre-inoculation treatments/manipulations**

### **2.2.1 In utero $LT\beta$ R-Ig treatment**

To create mice deficient in Peyer's patches (PPs), inguinal lymph nodes (ILNs) and many other lymphoid tissues, timed pregnant C57BL/Dk mice were given a single intravenous (i.v.) injection of 100  $\mu$ g of a fusion protein containing the soluble lymphotoxin  $\beta$  receptor ( $LT\beta$ R) domain linked to the Fc portion of human immunoglobulin G1 ( $LT\beta$ R-Ig; Force *et al.*, 1995) on day 11.5 of gestation. This reagent was a kind gift from Jeffrey Browning (Biogene Inc, Cambridge, MA, USA).

### **2.2.2 $\gamma$ -irradiation and bone-marrow reconstitution**

Groups of  $LT\alpha^{-/-}$ ,  $LT\beta^{-/-}$  and C57BL/6 mice were  $\gamma$ -irradiated (950 rad) 24 h prior to bone-marrow reconstitution. Bone-marrow from the femurs and tibias of adult C57BL/6 mice was prepared as single-cell suspensions ( $3 \times 10^7 - 4 \times 10^7$  cells) in Hank's balanced salt solution (HBSS; Life Technologies, Paisley, UK). Recipient

mice were reconstituted with 0.1 ml bone-marrow by injection into the tail vein. Mice were inoculated with ME7 scrapie 5 weeks later.

### **2.2.3 Injection of Indian ink**

Groups of  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and C57BL/6 mice were injected with 0.5 ml of a 10% solution of Indian ink via the intra-peritoneal route as described (Marco *et al.*, 1992). The thoracic cavities of these mice were examined 4 - 7 h later.

## **2.3. Inoculation with the scrapie agent**

All pathogenesis studies in this thesis involved inoculation with the ME7 scrapie agent strain (section 2.3.1). Mice were inoculated via various routes: intra-cranial (section 2.3.2); intra-peritoneal (section 2.3.3); via scarification of the skin of the thigh (section 2.3.4) and orally (2.3.5). After inoculation mice were monitored and individuals showing adverse reactions were killed using a schedule 1 method. Mice were coded and assessed weekly for clinical signs of disease and killed at the endpoint of disease (section 2.3.6). Confirmation of diagnosis was obtained by histopathological scoring of vacuolation of the brain (section 2.3.7)

### **2.3.1 ME7 scrapie agent strain**

The scrapie agent strain ME7 was originally isolated from spleen tissue of a case of natural scrapie in a Suffolk sheep and passaged by intra-gastric inoculation into Moredun random-bred mice (Zlotnik & Rennie, 1963). This was then further passaged by intra-cranial inoculation in Moredun random-bred mice before being passaged nine times through C57BL/Dk mice at NPU. The ME7 strain of scrapie has been extensively used and results in well characterised prominent neuronal



vacuolation. Following peripheral inoculation (for example, intra-peritoneal, oral or via the skin) an initial phase of agent accumulation in peripheral lymphoid tissues has been found to be necessary for the pathogenesis of this scrapie agent strain (Brown *et al.*, 1999b; Fraser & Dickinson, 1970, , 1978; Fraser & Farquhar, 1987; Mabbott *et al.*, 2000a; Mabbott *et al.*, 2002; Mabbott *et al.*, 2000b; Mabbott *et al.*, 2003; Taylor *et al.*, 1996b). The ME7 scrapie agent strain is, therefore, ideal for use in addressing the aims of this thesis.

### **2.3.2 Intra-cranial inoculation**

Mice were anaesthetised with 3% fluorothane gas (delivered in oxygen) before inoculation. 20µl unspun brain homogenate from C57BL/Dk mice terminally affected with the ME7 scrapie strain was injected into the right mid temporal cortex using a 1 ml syringe with a 26 gauge needle. The needle was sheathed to expose 2 mm length to ensure accuracy. Doses used were 1.0% (wt/vol) and 0.01% (wt/vol) dilution of scrapie-affected brain homogenate in physiological saline as indicated.

### **2.3.3 Intra-peritoneal inoculation**

Mice were inoculated by injection into the upper flank with a 26 gauge needle. The dose used was 20 µl of a 1.0% (wt/vol) dilution of scrapie-affected brain homogenate.

### **2.3.4 Inoculation via scarification of the skin**

Mice were inoculated by scarification of the medial surface of the right thigh as previously described (Mohan *et al.*, 2004; Taylor *et al.*, 1996b). Approximately 1 cm<sup>2</sup> of hair was removed from the site of inoculation, then 24 h later a 23 gauge needle was used to make a 1 cm long abrasion of the upper epidermal layer of the skin. A 26

gauge needle was then used to place one droplet (~6 µl) of scrapie affected brain homogenate on the abrasion site, which was then worked in with sweeping strokes and the abrasion sealed with Op-Site (Smith & Nephew Medical Limited, Hull, UK). The dose used was a 1.0% (wt/vol) dilution of brain homogenate.

### **2.3.5 Oral inoculation**

Mice were fed individual food pellets doused with 50 µl of a 1.0% (wt/vol) dilution of brain homogenate as described (Mabbott *et al.*, 2003).

### **2.3.6 Determination of scrapie incubation period**

The time elapsed (in days) between the day of inoculation and the clinical endpoint of disease is termed the incubation period. The clinical endpoint of disease was determined by rating the severity of clinical signs of TSE disease exhibited by the mice. Clinical status was recorded from around day 120 post inoculation (depending on route of exposure) and was carried out by experienced animal technicians. Mice were assessed weekly on the same day each week and were given a clinical score as “unaffected”, “possibly affected” and “definitely affected” using standard criteria (pronounced spinal hunching, ataxia or paralysis and “freezing” behaviour or hyperactivity). Terminal stages of disease were also associated with weight-loss or urinary incontinence in some cases. The clinical endpoint of disease was defined in one of four ways:

1. The day on which a mouse received a second consecutive “definite” rating;
2. The day on which a mouse received a third “definite” rating within four consecutive weeks;
3. The day on which a mouse was culled *in extremis*;

4. The day on which a mouse was found dead in its cage having received a “definite” rating the previous week.

This system has been applied to a wide range of TSE experiments and has been in use at NPU for many years.

### **2.3.7 Lesion profiles**

Whole brains were fixed in formal saline and cut into five defined areas (Fraser & Dickinson, 1968) and embedded in paraffin wax. 6 µm thick sections were cut and stained with haemotoxylin and eosin. These sections were then examined and the degree of scrapie-specific vacuolation in the neuropil scored by experienced histology staff. Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (G1-G9) and white matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of superior peduncles; W3, cerebral peduncles.

## **2.4. Ex vivo analysis of tissues**

### **2.4.1 Immunohistochemical and immunofluorescent histochemical analysis of frozen tissues**

Spleen or mesenteric lymph nodes were snap frozen at the temperature of liquid nitrogen. Small intestine was divided into three roughly equal parts, gently squeezed to remove gut contents, coiled and embedded in Tissue-Tek® O.C.T. Compound™ (Bayer PLC, Newbury, UK) and snap frozen. Serial frozen sections (10 µm in



Target	Antigen/residue	Antibody/clone/lectin	Source/Ref
FDCs	C4	mAb FDC-M2	AMS Biotechnology, Oxon, UK, (Taylor <i>et al.</i> , 2002)
FDCs	CD35/CD21	mAb 7G6	BD Pharmingen
FDCs	CD35	mAb 8C12	BD Pharmingen
FDCs	C3	mAb RMC7H8	Connex
B lymphocytes	CD45R (B220)	mAb RA3 6B2	Caltag, Towcester, UK
Germinal centre B lymphocytes	galactosyl ( $\beta$ 1-3) N-acetylgalactosamine	Peanut agglutinin (PNA)	Vector Labs, (Rose <i>et al.</i> , 1980)
T lymphocytes	CD3 $\epsilon$	mAb145-2C11	BD Pharmingen
Lymphatic vessels	Hyaluronic acid receptor	Lyve-1	Upstate, Dundee, UK

**Table 2.1. Primary antibodies and lectins used for immunohistochemical and immunofluorescent analysis of frozen spleen, mesenteric lymph node and small intestine tissue.** mAb = monoclonal antibody.

thickness) were cut on a cryostat. Sections were then analysed using the antibodies and lectins detailed in Table 2.1.

For light microscopy, following the addition of primary antibody, biotin conjugated species specific secondary antibodies (Stratech, Soham, UK) were applied followed by alkaline phosphatase coupled to the avidin/biotin complex (Vector Laboratories, Peterborough, UK). Vector Red (Vector Labs) was used as a substrate. Sections were counterstained with haematoxylin to distinguish cell nuclei and mounted using Pertex (CellPath, Powys, UK) mounting medium.

For fluorescent microscopy, following the addition of primary antibody, species specific secondary antibodies coupled to Alexa dyes (Invitrogen, Paisley, UK) 488 (green) or 594 (red) were used. Sections were mounted using fluorescent mounting medium (Dako, Ely, UK) and examined using a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK).

#### **2.4.2 Immunohistochemical and immunofluorescent histochemical analysis of paraffin embedded tissues**

Small intestines or brains were fixed in 2% periodate-lysine-paraformaldehyde (0.1 M periodate; 0.075 M D-L lysine; 2% paraformaldehyde in 0.05 M phosphate buffer pH 7.4) and embedded in paraffin wax. Sections (6  $\mu$ m in thickness) were cut on a microtome and deparaffinised. Small intestine sections were then analysed using the antibodies and lectins detailed in Table 2.2. Brain tissue sections were analysed using the antibodies detailed in Table 2.3.

Target	Antigen/residue	Antibody/clone/ Lectin	Source/Ref	Pre treatment
FDCs	CD35/CD21	mAb 7G6	BD Pharmingen	TRS
B lymphocytes	CD45R (B220)	mAb RA3 6B2	Caltag	N/A
Germinal centre B lymphocytes	galactosyl ( $\beta$ 1-3) N- acetylgalactosamine	Peanut agglutinin (PNA)	Vector Labs, (Rose <i>et al.</i> , 1980)	TRS
Prion protein	PrP	polyclonal Ab 1B3	(Farquhar <i>et al.</i> , 1989)	HW+FA
M cells	$\alpha$ -L-fucose	<i>Ulex europaeus</i> agglutinin (UEA)	Vector Labs	N/A
Nerves	S-100	Polyclonal anti-S- 100	Dako	TRS

**Table 2.2- Primary antibodies and lectins used for immunohistochemical and immunofluorescent analysis of paraffin embedded small intestine tissue.** mAb = monoclonal antibody. TRS = sections were microwaved (low power, 650W microwave for 13 mins with periodic agitation) in Target Retrieval Solution (Dako) prior to application of primary antibody or lectin. HW+FA = sections were immersed for 30 min in water at 55 °C and then in 98 % formic acid for 5 min prior to application of primary antibody. N/A = not applicable.



Target	Antigen/residue	Antibody/clone/ lectin	Source/Ref	Pre treatment
Prion protein	PrP	mAb 6H4	Prionics, Zurich, Switzerland	HA+FA
Activated astrocytes	Glial fibrillary acidic protein (GFAP)	Polyclonal anti- GFAP	Dako	N/A

**Table 2.3- Primary antibodies used for immunohistochemical analysis of paraffin embedded brain tissue.** mAb = monoclonal antibody. HA+FA = sections were treated with hydrated autoclaving (15 min, 121 °C, hydration) and then in 98 % formic acid for 5 min prior to application of primary antibody. N/A = not applicable

For light microscopy, following the addition of primary antibody, biotin conjugated species specific secondary antibodies were used followed by alkaline phosphatase coupled to the avidin/biotin complex (Vector Labs). Vector Red was used as a substrate. Sections were counterstained with haematoxylin to distinguish cell nuclei and mounted using Pertex mounting medium.

For fluorescent microscopy, following the addition of primary antibody, species specific secondary antibodies coupled to Alexa dyes 488 (green) or 594 (red) were used. Sections were mounted using fluorescent mounting medium and examined using a confocal microscope.

#### **2.4.3 Detection of PrP<sup>Sc</sup> by immunoblotting**

PrP<sup>Sc</sup> was enriched from tissue homogenates by sodium phosphotungstic acid (NaPTA) precipitation (Wadsworth *et al.*, 2001). Briefly, tissue homogenates were prepared from spleen fragments (approximately 20 mg) or MLNs or ILNs (approximately half the total from each mouse assayed) and centrifuged for 10 mins at 2000 x g. Supernatants were removed, mixed with an equal volume of 2 % (wt/vol) N-laurylsarcosine in Tris pH 7.4 and incubated for 10 mins at 37 ° C with constant agitation. Samples were divided into two equal parts. To one half, 2 µl of 20 mg/ml proteinase K (VWR, Lutterworth, UK) was added and the contents were incubated for 60 mins at 37°C with constant agitation. The other half was held at 4°C. Following incubation, to each fraction 4% (wt/vol) NaPTA, pH 7.4, containing 170 mM MgCl<sub>2</sub> was added to achieve a final concentration of 0.3% NaPTA. Samples were incubated for 30 mins at 37°C with constant agitation followed by centrifugation at 13000 x g for 30 mins. Pellets were washed with 200 µl 0.1%

(wt/vol) N-lauroylsarcosine in Tris pH 7.4 plus 100  $\mu$ l 250 mM EDTA and centrifuged at 13000 x g for 30 mins. Pellets were resuspended and assayed for protein concentration using a BCA protein assay (Pierce, Chester, UK) and adjusted to 0.5 mg/ml where possible. Samples were then subjected to electrophoresis through sodium dodecyl sulphate 12% polyacrylamide gels (Invitrogen) and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hemel Hemstead, UK) by semidry blotting. PrP was detected with the PrP-specific mouse monoclonal antiserum 8H4 which bind amino acids 147-164 of murine PrP. This epitope is present on PrP<sup>Sc</sup> truncated by PK treatment (Zanusso *et al.*, 1998). Horse radish peroxidase-conjugated goat anti-mouse antiserum (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa, USA) was then applied and bound horse radish peroxidase activity was detected with Supersignal West Dura Extended Duration Substrate (Pierce).

#### **2.4.4 Morphometric analysis**

The number of isolated lymphoid follicles (ILFs) was counted microscopically using CD45R expression for identification. The maturity of the ILFs was then determined depending on the presence of CR1/CR2 expressing FDCs. An image of each mature ILF was captured using a confocal microscope (Zeiss) and the area covered by the CR1/CR2 expressing cells was measured using Image Pro Plus (Media Cybernetics, Wokingham, UK).



#### **2.4.5 Measurement of faecal IgA levels by enzyme-linked immunosorbant assay (ELISA)**

Faecal pellets were collected and faecal IgA levels were determined by ELISA as previously described (McDonald *et al.*, 2005). Briefly, 10% faecal homogenates were prepared in carbonate-bicarbonate coating buffer (Sigma) and centrifuged for 10 min at 12,000 x *g*. Supernatant was removed and adsorbed to flat-bottomed high binding microwell plates (Costar®, High Wycombe, UK) for 18-24 hrs at 4 °C. Plates were then blocked with 0.01 M PBS (pH 7.5) containing 5 % bovine serum albumin (BSA). Bound IgA was then detected using alkaline phosphatase conjugated goat anti-mouse IgA (Southern Biotechnology, AL, USA). Bound alkaline phosphatase activity was measured by incubation with p-nitrophenyl phosphate liquid substrate (Sigma). Optical density (OD) was measured at 450 nm using a V-max kinetic microplate reader (Molecular Devices, CA, USA). Faecal pellets from C57BL/6 wild-type mice, C57BL/Dk control mice and SCID mice were used as reference controls.

### **2.5. Statistical analysis**

#### **2.5.1 Statistical analysis**

Data are presented as mean  $\pm$  standard error of the mean (S.E.M.). Significant differences between samples in different groups were sought by one-way analysis of variance (ANOVA) using Minitab software. Values of  $p < 0.05$  were accepted as significant.

# 3

## Characterisation of isolated lymphoid follicles (ILFs)

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### **3.1 Abstract**

The presence of IgA in the intestines of mice lacking Peyer's patches (PPs) has led to the recent discovery of intestinal lymphoid tissues known as isolated lymphoid follicles (ILFs). ILFs have previously been found in greater numbers in mice lacking PPs compared to immunocompetent controls. In this chapter ILFs were investigated in the small intestines of mice that lack PPs either due to *in utero* lymphotoxin- $\beta$  receptor (LT $\beta$ R) signalling blockade or due to genetic deficiency of either LT $\alpha$  or LT $\beta$ . In both cases, follicular dendritic cell (FDC) presence was found to be a consistent marker of ILF maturity. Increased numbers of larger mature ILFs (mILFs) were found in mice lacking PPs compared to relevant control mice, and these mILFs were found to adequately compensate for the lack of PPs in terms of the total area covered by FDCs in the small intestine. mILFs were also found to contain T lymphocytes, germinal centre B lymphocytes, lymphatic vessels and the follicle associated epithelium (FAE) overlying the mILFs contained M cells. The detection of IgA in the intestines of mice lacking PPs or both PPs and mesenteric lymph nodes (MLNs) correlated with the presence of mILFs. Taken together these data clearly demonstrate that mILFs are fully functional gut-associated lymphoid tissues. As FDCs are important for the peripheral pathogenesis of TSEs these data also suggest that mILFs might be an important additional site of TSE agent neuroinvasion.



### **3.2 Introduction**

The gastrointestinal tract is a major interface between the host and potential pathogens. Therefore, the immunological components that defend the gastrointestinal tract have been extensively studied. Intestinal immune responses are initiated in highly organised lymphoid tissues such as the Peyer's patches (PPs), mesenteric lymph nodes (MLNs), appendix and tonsils (collectively known as gut associated lymphoid tissues; GALT). These responses are executed by loosely distributed effector cells such as intra-epithelial lymphocytes and lamina propria lymphocytes. One of the major defences of the immune system against invading pathogens is the secretion into the intestine of secretory immunoglobulin (Ig) A. The production of IgA is thought to be initiated in PPs and effected by plasma cells in the lamina propria of villi (MacPherson *et al.*, 2001). The finding that IgA is present in the intestines of mice which have been manipulated to lack PPs (Yamamoto *et al.*, 2000) has led to the recent discovery of additional organised lymphoid tissues within the intestine, known as isolated lymphoid follicles (ILFs; Hamada *et al.*, 2002).

ILFs are present in both the small and large intestines of mice (Hamada *et al.*, 2002; Kweon *et al.*, 2005; Lorenz & Newberry, 2004) rabbits (Keren *et al.*, 1978), guinea pigs (Rosner & Keren, 1984) and humans (Moghaddami *et al.*, 1998). The term ILF incorporates a plethora of structures ranging from a loose cluster of B lymphocytes at the base of a villus to highly organised structures the width of one or more villi. ILFs are small solitary follicles unlike PPs which are an aggregation of several large follicles. ILFs have been found to contain T lymphocytes, germinal centre B lymphocytes, dendritic cells and epithelia overlying ILFs contain M cells (Hamada *et*

*al.*, 2002; Lorenz *et al.*, 2003), but whether these structures also contain follicular dendritic cells (FDCs) is not known.

Since lymphotoxin (LT)  $\alpha_1\beta_2$  signalling through the LT $\beta$  receptor (LT $\beta$ R) during gestation has been shown to be crucial for the formation of PPs (Banks *et al.*, 1995; Honda *et al.*, 2001; Koni *et al.*, 1997; Rennert *et al.*, 1996; Togni *et al.*, 1994; Yoshida *et al.*, 1999), it follows that mice in which this signalling has been interrupted during gestation will lack PPs. Studies have shown that mice which lack PPs but have functional lymphotoxin signalling as adults have increased numbers of ILFs in both the large and small intestines (Hamada *et al.*, 2002; Kweon *et al.*, 2005; Lorenz *et al.*, 2003).

As FDCs are important for efficient TSE agent neuroinvasion from the gastrointestinal tract after oral inoculation (Mabbott *et al.*, 2003), ILFs were examined for the presence of FDCs. FDC presence within ILFs might identify another potentially important site of TSE agent neuroinvasion within the gastrointestinal tract.

### **3.3 Results**

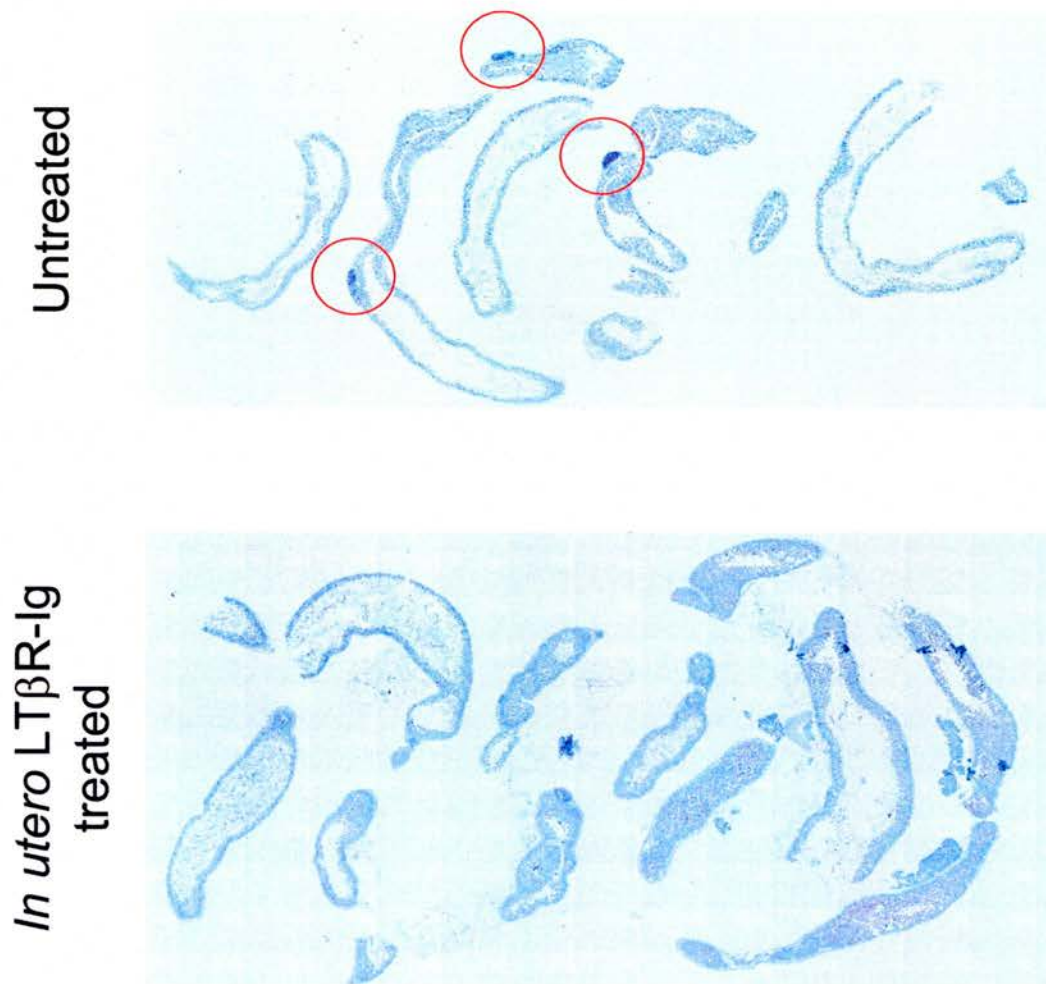
#### **3.3.1 *In utero* LT $\beta$ R-Ig treatment of mice induces the maturation of ILFs**

To temporarily block the LT $\beta$ R signalling pathway *in utero*, pregnant mice were given a single i.v. injection of 100  $\mu$ g of LT $\beta$ R-Ig (section 2.3.1; Force *et al.*, 1995) on day 11.5 of gestation. In accordance with previous studies, PPs in the intestines of progeny LT $\beta$ R-Ig treated mice were macroscopically absent (Rennert *et al.*, 1996). Intestines from age-matched, untreated, immunocompetent C57BL/Dk (control) mice contained approximately 7 PPs (Fig 3.1). Microscopical analysis revealed the presence of both PPs and ILFs in the intestines of untreated control mice, whereas only ILFs were found in the intestines of mice treated *in utero* with LT $\beta$ R-Ig (termed PP-deficient C57BL/Dk mice; Fig 3.2)

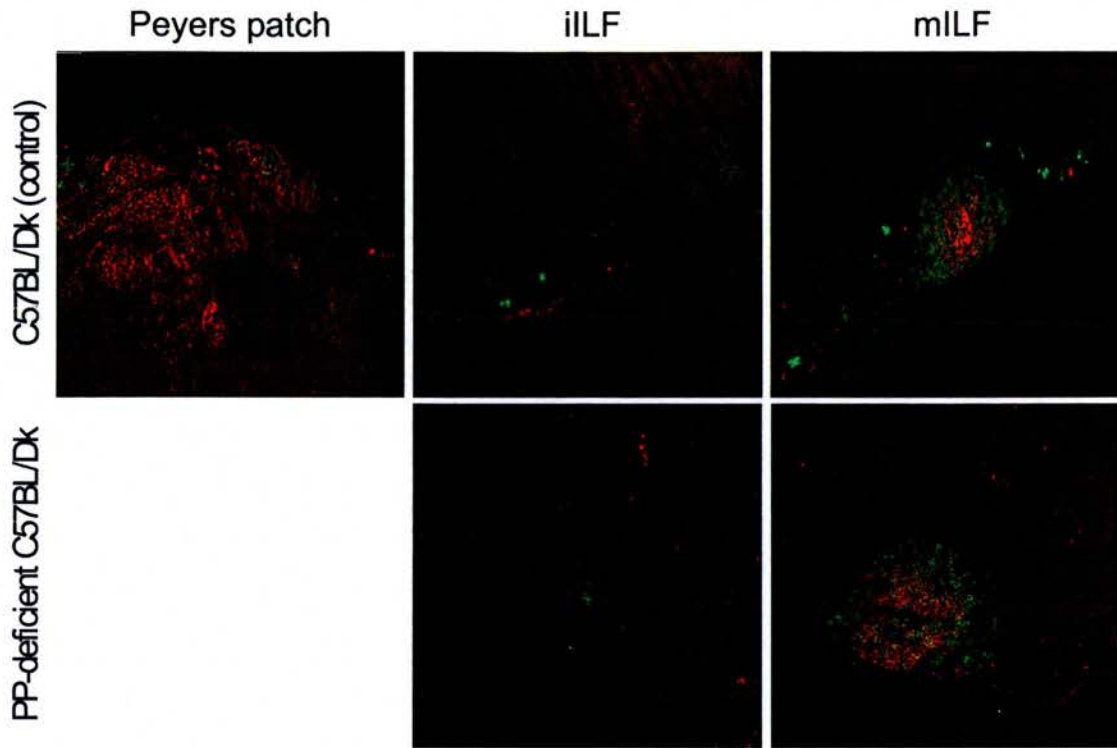
#### **3.3.2 Mature ILFs contain FDCs**

The maturity of ILFs has previously been defined depending on the density of component cells and location of the structure, such that loosely clustered B220-positive cells located at the base of a villus were considered to be immature ILFs (iILFs) whereas well organised, nodular structures with the same or greater width than one villus were considered to be mature (mILFs; Lorenz *et al.*, 2003). Here, double immunostaining of B lymphocytes (CD45R-positive cells) and FDCs (CD21/35-positive cells) on the same tissue section revealed that the presence of FDCs could also be used to reliably determine ILF maturation status. Experiments showed that mILFs contained FDCs whereas iILFs did not (Fig 3.2). A major function of FDCs is to trap and retain antigen on their surfaces for presentation to B





**Figure 3.1- Effect of *in utero* LTβR-Ig treatment on the development of Peyer's patches (PPs).** Small intestine from untreated, immunocompetent, C57BL/Dk (control) mice (top) showed the presence of PPs (red circles). Small intestine from progeny mice treated *in utero* with LTβR-Ig showed the macroscopic absence of PP development. Sections were stained with CD45R-specific antiserum B220 to detect B lymphocytes (red) and counterstained with haematoxylin (blue). Original magnification X40.



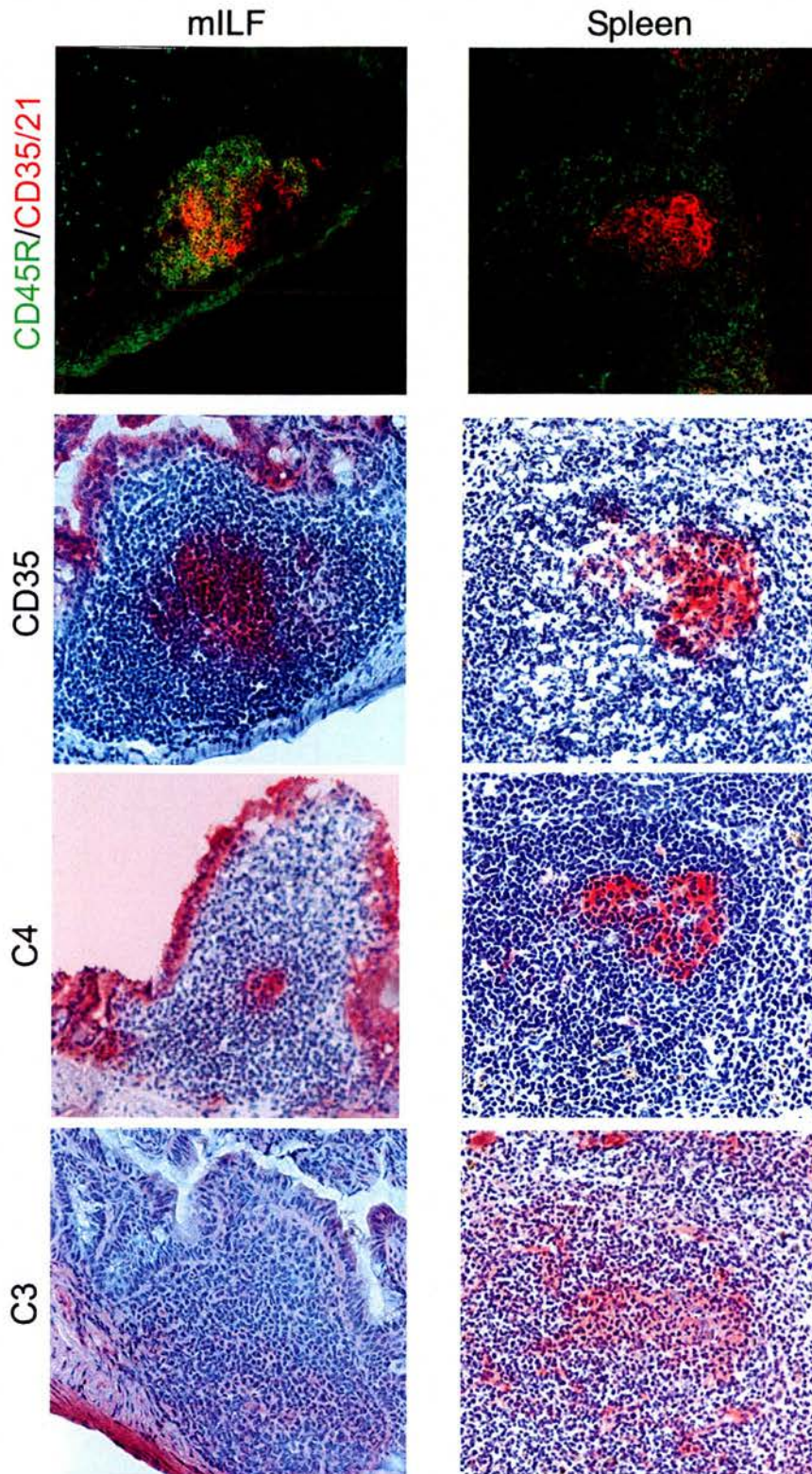
**Figure 3.2- Effect of *in utero* LT $\beta$ R-Ig treatment on the development of lymphoid tissue in the small intestine.** Sections were stained with the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green) and 7G6 monoclonal antiserum to detect CD21/35 expressing follicular dendritic cells (FDCs; red). Tissues from untreated, immunocompetent, C57BL/Dk (control) mice (top row) showed the presence of B lymphocyte and FDC containing Peyer's patches (left column), B lymphocyte containing immature isolated lymphoid follicles (iILFs; middle column) and B lymphocyte and FDC containing mature ILFs (mILFs; right column). Tissues from progeny mice treated *in utero* with LT $\beta$ R-Ig (lower row) showed the presence of only iILFs (middle column) and mILFs (right column). Original magnification X200.

lymphocytes. The attachment of antigen to FDCs is mediated via interactions between complement components and complement receptors (CR) on the surface of FDCs (Berg *et al.*, 1995). Complement components C3 and C4, in association with CR1 (CD35) and CR2 (CD21) expressing FDCs, were detected within mILFs (Fig 3.3) but not in iILFs (not shown). Taken together these data clearly demonstrate that mILFs in the intestines of PP-deficient C57BL/Dk mice contain immune complex trapping FDCs.

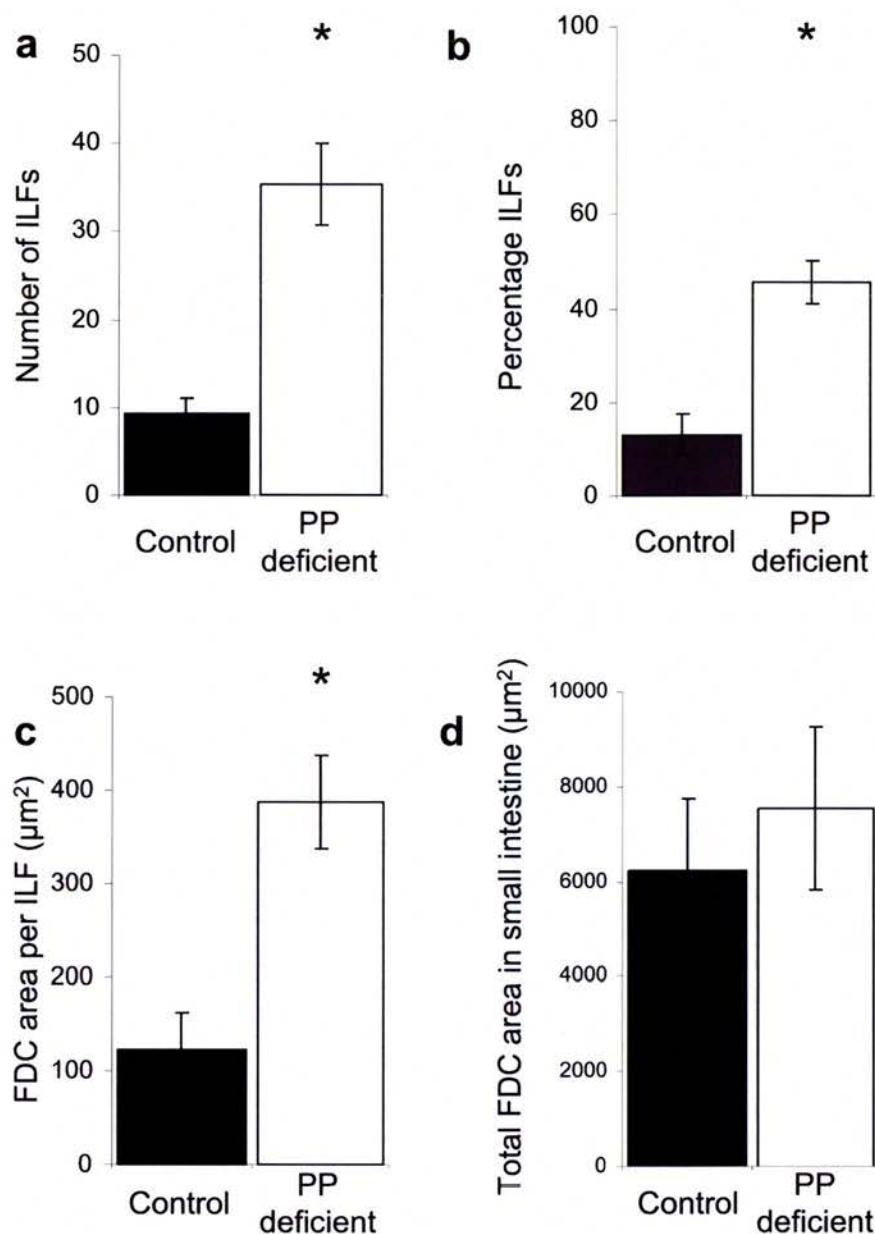
### **3.3.3 mILFs can compensate for PP deficiency**

The total number of ILFs in the small intestine of PP-deficient mice was significantly greater than in control mice ( $p \leq 0.001$ ; Fig 3.4a). Approximately  $9 \pm 2$  ILFs were detected in the intestines of control mice, whereas those from PP-deficient mice contained  $35 \pm 5$  ILFs. Although the small intestines of both groups of mice were found to contain both mILFs and iILFs (Fig 3.2), a greater proportion of ILFs in PP-deficient mice were mature when compared to those in control mice ( $\sim 46\%$  and  $\sim 15\%$  respectively;  $p \leq 0.001$ ; Fig 3.4b). When FDC area per mILF was taken as a measure of mILF size, PP-deficient mice were found to have significantly larger mILFs when compared to control mice ( $p \leq 0.001$ ; Fig 3.4c). Further investigation revealed that the greater number of larger mILFs present in PP-deficient mice compensated for the PP deficiency in terms of the total area covered by FDCs in the small intestine. The total area covered by the FDCs in all the mILFs in PP-deficient mice was found to be equivalent to the total area covered by the FDCs in all the PPs and the few mILFs found in control mice ( $p \leq 0.572$ ; Fig 3.4d).





**Figure 3.3- Mature isolated lymphoid follicles (mILFs) contain functional follicular dendritic cells (FDCs).** Sections of small intestine (left) or spleens (right) sections were stained with the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green; top row) and CD21/35-specific (red; top row) and CD35-specific (red; 2<sup>nd</sup> row) monoclonal antisera to detect FDCs and monoclonal antisera specific for complement components C4 (FDC-M2; red; 3<sup>rd</sup> row) and C3 (red; lower row). Original magnification X200.



**Figure 3.4- Enumeration of isolated lymphoid follicles (ILFs) and follicular dendritic cells (FDCs) in the small intestine of Peyer's patch (PP) -deficient C57BL/Dk mice and immunocompetent C57BL/Dk (control) mice. a.** Intestines from PP-deficient mice contained significantly more (~ 3 fold more) ILFs than intestines from control mice. **b.** Intestines from PP-deficient mice were found to have a significantly higher percentage (46 %) of mature ILFs (mILFs) when compared to control mice (13 %). **c.** FDC area per mILF was taken as a measure of mILF size. PP-deficient mice contained significantly larger mILFs when compared to control mice. **d.** The total area covered by FDCs in the mILFs in PP-deficient mice was equivalent to the total area covered by FDCs from all the PPs and mILFs in control mice. Each bar represents the mean  $\pm$  S.E.M. for each group of mice ( $n = 12$  for PP deficient mice,  $n = 11$  for control mice). \* indicates a  $p$  value of  $\leq 0.05$  when compared to control mice.

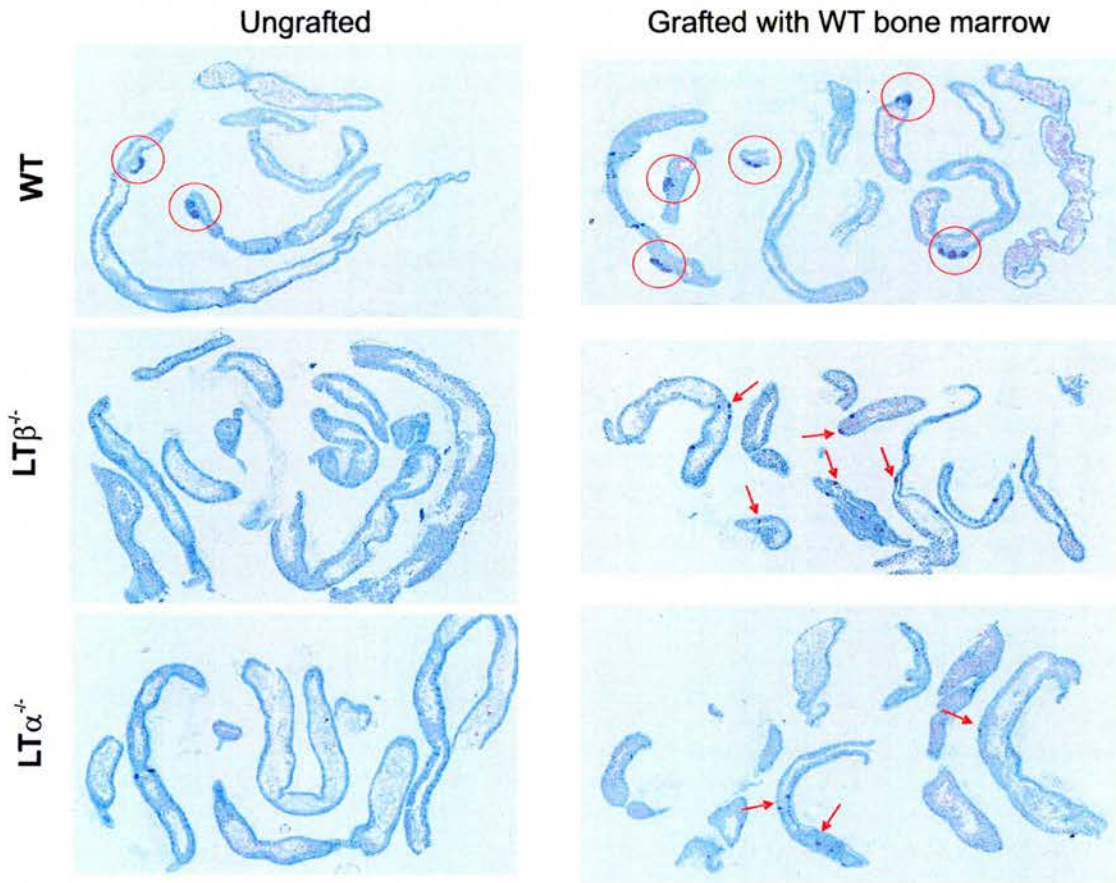


### **3.3.4 LT-expressing bone marrow-derived cells induce the development of ILFs in LT deficient mice**

LT-signalling is required for the formation of PP and ILFs, hence both are absent in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice (Fig 3.5; Hamada *et al.*, 2002; Koni *et al.*, 1997; Lorenz *et al.*, 2003; Togni *et al.*, 1994). The source of the LT required for the formation of ILFs has been shown to be bone marrow derived cells, as reconstitution of  $LT\alpha^{-/-}$  mice with immunocompetent wild-type (WT) bone marrow restores the formation of ILFs (Fig 3.5; Lorenz *et al.*, 2003). Data presented here show, in addition, that WT bone marrow reconstitution stimulates the formation of ILFs in  $LT\beta^{-/-}$  mice. However, WT bone marrow reconstitution does not restore the formation of PPs in either  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice (Fig 3.5; Koni & Flavell, 1998; Mariathasan *et al.*, 1995). Both PPs and ILFs are found in the intestines of WT mice before and after reconstitution with WT bone marrow (Fig 3.5).

The total number of ILFs present in the small intestines of WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice before and after  $\gamma$ -irradiation and WT bone marrow reconstitution was determined (Fig 3.6). ILFs were present in the small intestine of WT mice prior to  $\gamma$ -irradiation and WT bone marrow reconstitution, whereas none were detected in either  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice at this time. By 35 days after bone marrow reconstitution the number of ILFs in the small intestines of recipient WT mice (termed WT->WT mice) had significantly declined ( $p \leq 0.004$ ,  $n = 4$  when compared to the number in WT mice pre-bone marrow reconstitution) and none were detected in recipient  $LT\alpha^{-/-}$  mice (termed WT-> $LT\alpha^{-/-}$  mice) or  $LT\beta^{-/-}$  mice (termed WT-> $LT\beta^{-/-}$  mice). However, by 105 days after WT bone marrow reconstitution, the



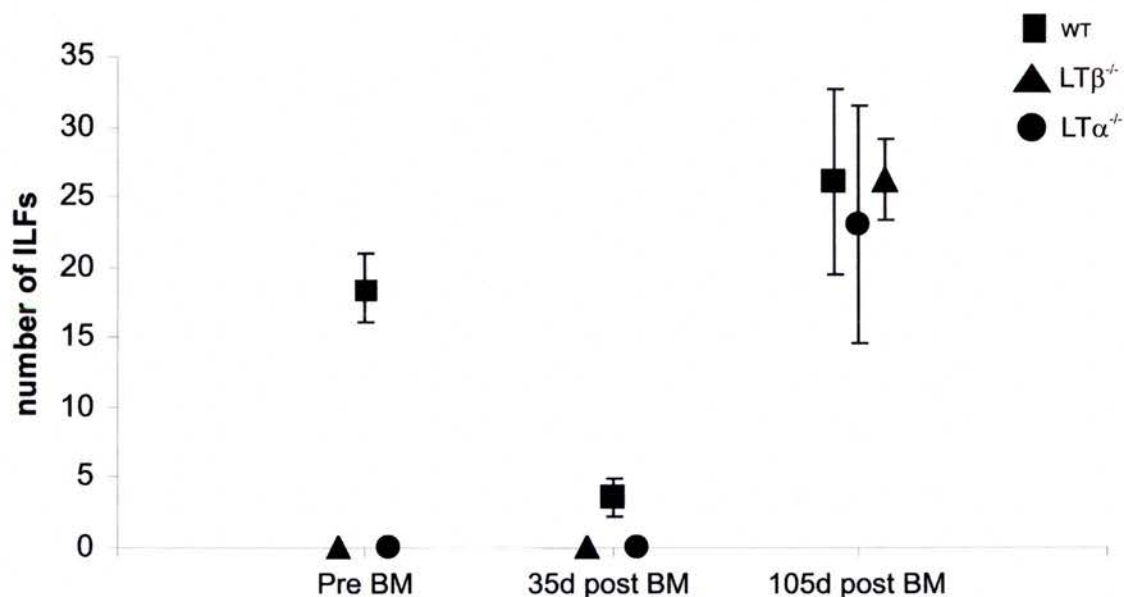


**Figure 3.5- Effect of reconstitution with wild-type (WT) bone marrow on the development of Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) in recipient WT mice (WT->WT mice), lymphotoxin  $\alpha$  deficient mice (WT->LT $\alpha$ <sup>-/-</sup> mice) and lymphotoxin  $\beta$  deficient mice (WT->LT $\beta$ <sup>-/-</sup> mice).** Small intestine from unreconstituted mice (left column) and bone marrow reconstituted mice (right column) were stained with the CD45R-specific antiserum B220 to detect B lymphocytes (red) and counterstained with haematoxylin (blue). In WT mice PP development (red circle) was unaffected by bone marrow reconstitution and ILFs were macroscopically invisible. In both LT $\alpha$ <sup>-/-</sup> mice (middle row) and LT $\beta$ <sup>-/-</sup> mice (lower row), PPs were not detectable prior to (left column), or after (right column) bone marrow reconstitution. In LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice ILFs were macroscopically undetectable in unreconstituted mice but visible in bone marrow reconstituted animals (arrows, right column, middle and lower panels). Original magnification X40.

number of ILFs in the intestines of WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice was comparable to the number detected in WT mice before  $\gamma$ -irradiation and WT bone marrow reconstitution ( $p \leq 0.560$ ,  $n = 6$ ;  $p \leq 0.779$ ,  $n = 6$ ; and  $p \leq 0.185$ ,  $n = 5$ ; respectively, when compared to the number in WT mice before bone marrow reconstitution; Fig 3.6).

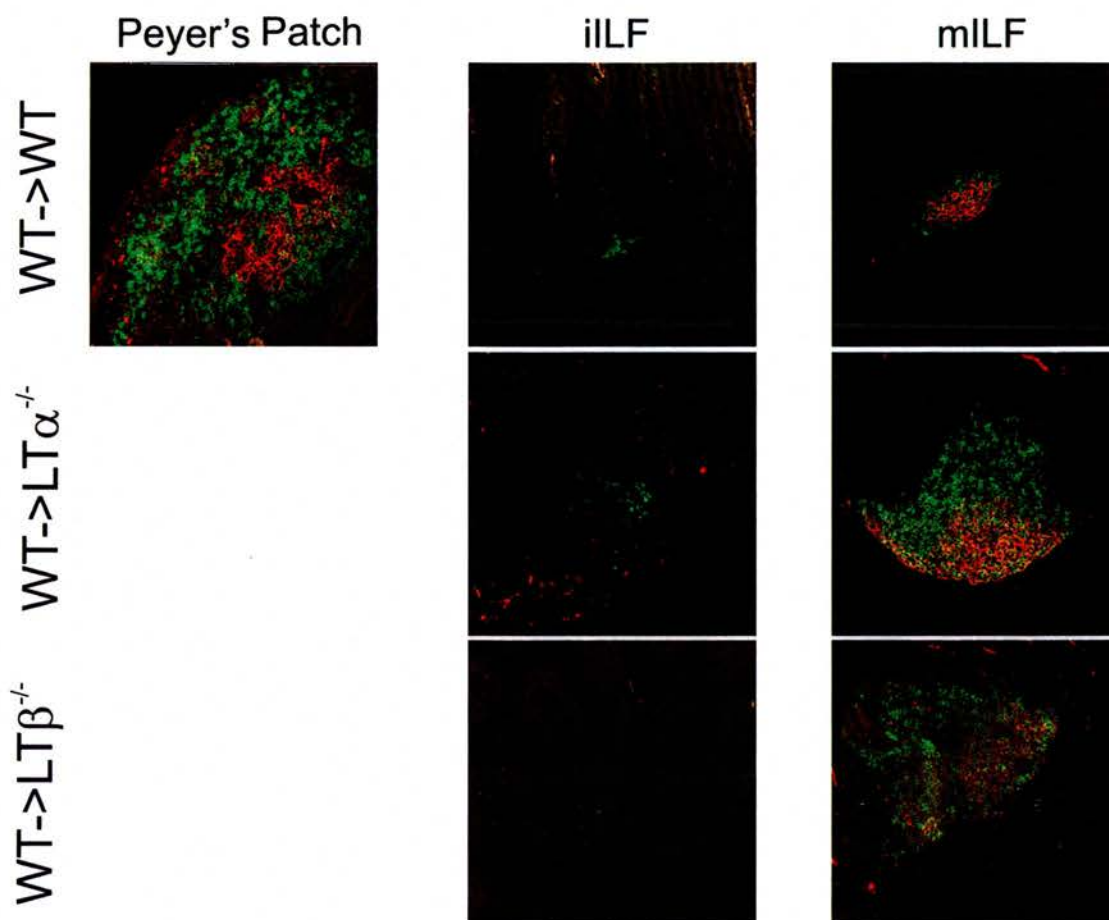
### **3.3.5 mILFs in WT bone marrow reconstituted LT deficient mice contain FDCs**

Intestines from WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice were analysed 105 days after  $\gamma$ -irradiation and reconstitution with WT bone marrow. In each of the three groups of mice, both mILFs and iILFs were detected (Fig 3.7). Consistent with data presented in Fig 3.3, mILFs were found to contain functional CR1/CR2-expressing FDCs (Fig 3.8). Although the numbers of ILFs in the small intestines of WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice were found to be similar (Fig 3.9a), the percentage of mILFs was significantly higher in WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice when compared to WT->WT mice (55 %,  $p \leq 0.028$ ,  $n = 6$  and 62 %,  $p \leq 0.002$ ,  $n = 5$  respectively, when compared to 30 %,  $n = 6$  in WT->WT mice; Fig 3.9b). The mILFs in the intestines of WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice were found to be significantly larger than those of WT->WT mice ( $p \leq 0.009$ ,  $n = 6$ , and  $p \leq 0.026$ ,  $n = 5$  respectively when compared to WT->WT mice ( $n = 6$ ; Fig 3.9c). The total area occupied by FDCs in the small intestines of all three types of mice was found to be equivalent ( $p \leq 0.099$ ,  $n = 6$ , and  $p \leq 0.162$ ,  $n = 5$ , in WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively when compared to WT->WT; Fig 3.13d). This suggests that analogous to the results found in PP-deficient C57BL/Dk mice (section 3.3.1), mILFs are capable of compensating for the PP

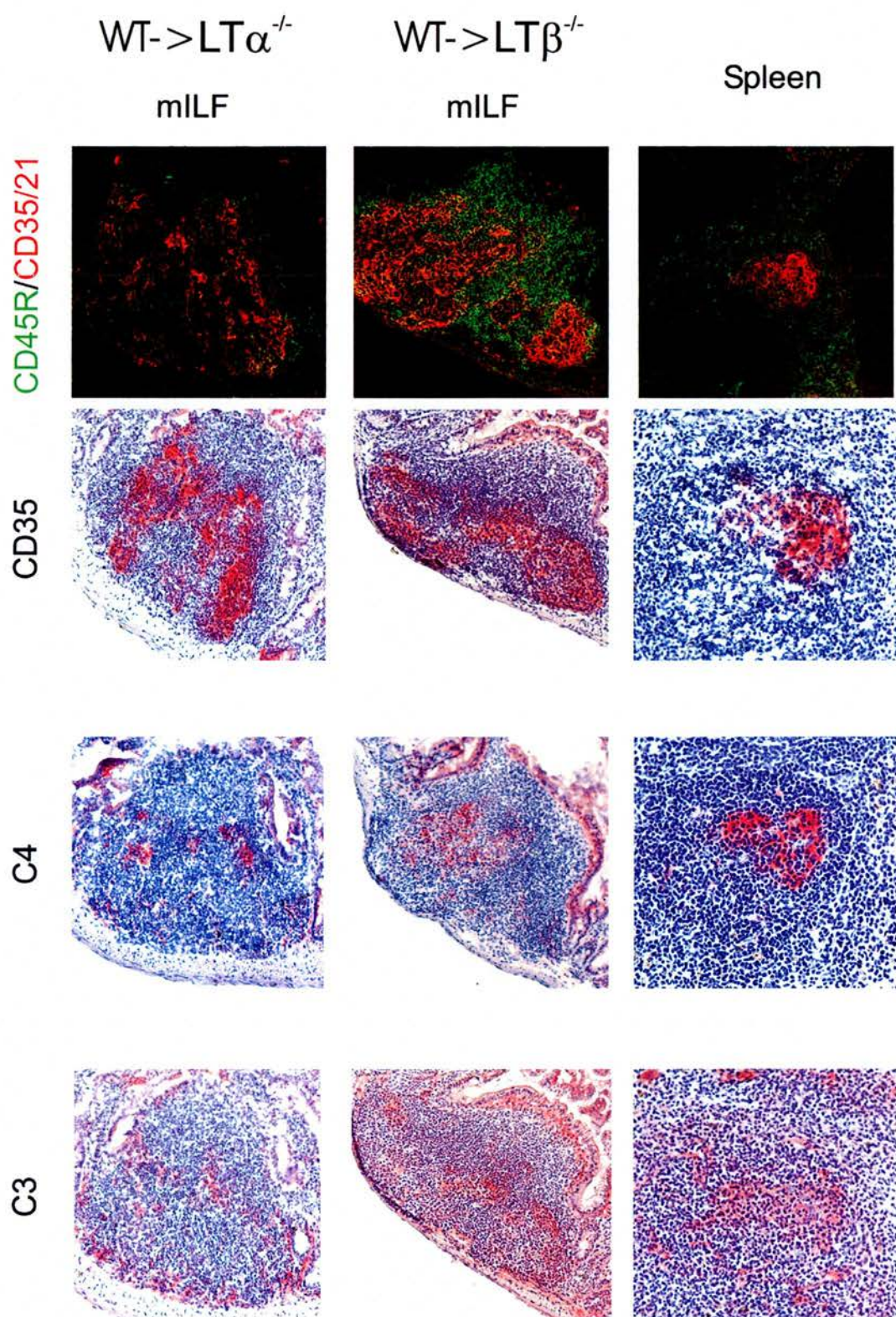


**Figure 3.6- Numbers of isolated lymphoid follicles (ILFs) in the small intestine of wild-type (WT) mice, lymphotoxin  $\alpha$  deficient ( $LT\alpha^{-/-}$ ) mice and lymphotoxin  $\beta$  ( $LT\beta^{-/-}$ ) mice pre and post WT bone marrow reconstitution.** Prior to bone marrow reconstitution ILFs were present in the intestines of WT mice (squares) but not in either  $LT\alpha^{-/-}$  mice (circles) or  $LT\beta^{-/-}$  mice (triangles). 35 days after bone marrow reconstitution the number of ILFs in WT->WT mice had fallen and none were detectable in either WT-> $LT\alpha^{-/-}$  mice or WT-> $LT\beta^{-/-}$  mice. 105 days after bone marrow reconstitution the number of ILFs in WT->WT mice, WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice was comparable to unreconstituted WT mice. Each point represents the mean  $\pm$  S.E.M. for each group of mice.



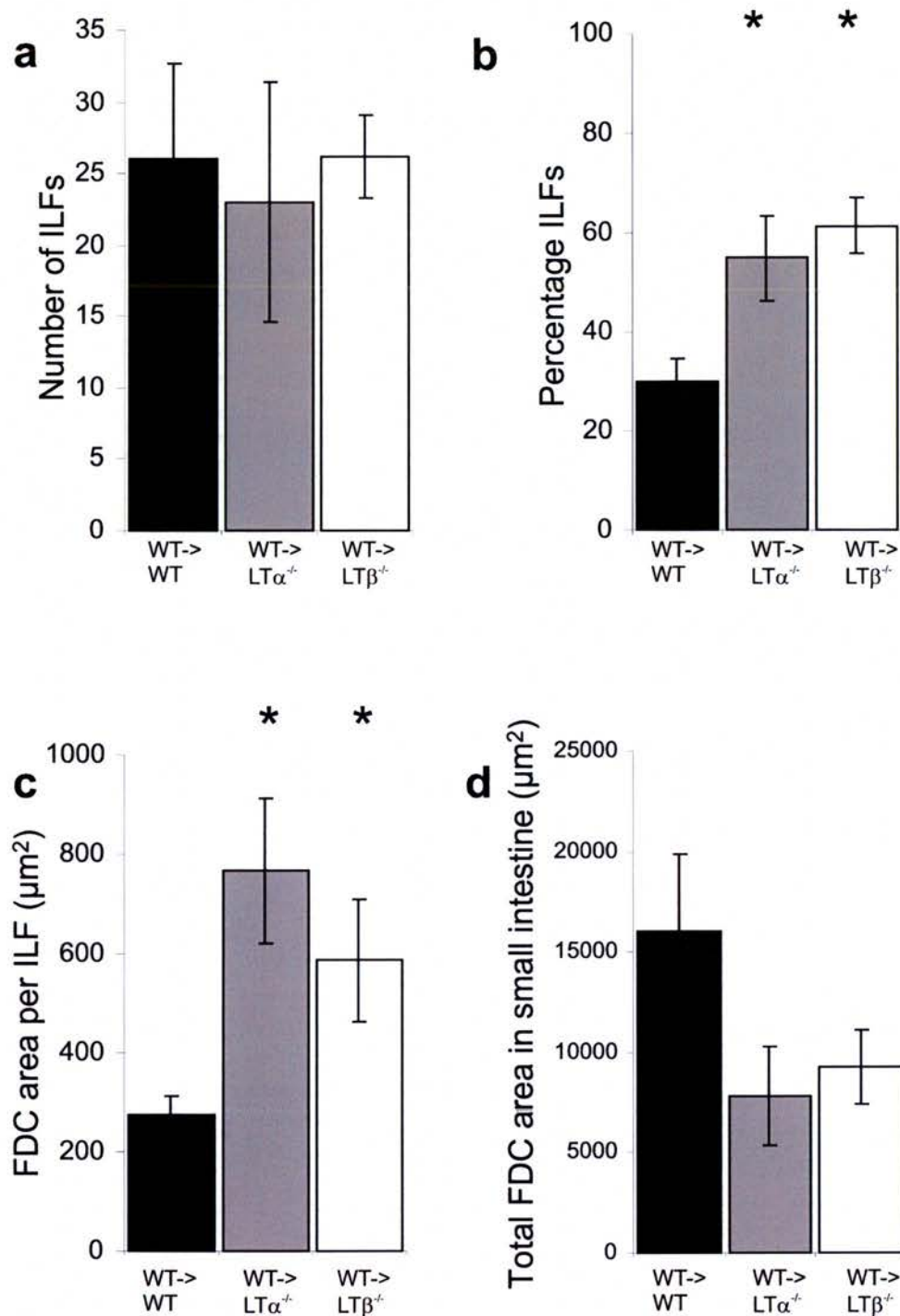


**Figure 3.7- Lymphoid tissue microarchitecture in the small intestine of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively).** Sections were stained using the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green) and CD21/35 specific antiserum to detect follicular dendritic cells (FDCs; red). Tissues from WT->WT mice (top row) showed the presence of B lymphocyte and FDC containing Peyer's patches (PPs; left column), B lymphocyte containing immature isolated lymphoid follicles (iILFs) (middle column) and B lymphocyte and FDC containing mature ILFs (mILFs; right column). Tissues from both WT->LT $\alpha^{-/-}$  mice (middle row) and WT->LT $\beta^{-/-}$  mice (lower row) showed the presence of B lymphocyte containing iILFs (middle column) and B lymphocyte and FDC containing mILFs (right column). Original magnification X200.



**Figure 3.8- Mature isolated lymphoid follicles (mILFs) contain functional follicular dendritic cells (FDCs).** Sections of small intestine from wild type bone marrow reconstituted lymphotoxin  $\alpha$  deficient mice (WT->LT $\alpha^{-/-}$  mice; left column) and WT->LT $\beta^{-/-}$  mice (middle column) or spleens (right column) were stained with the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green; top row) and CD21/35-specific (red; top row) and CD35-specific (red; 2<sup>nd</sup> row) monoclonal antisera to detect FDCs and monoclonal antisera specific for complement components C4 (FDC-M2; red; 3<sup>rd</sup> row) and C3 (red; lower row). Original magnification X200.





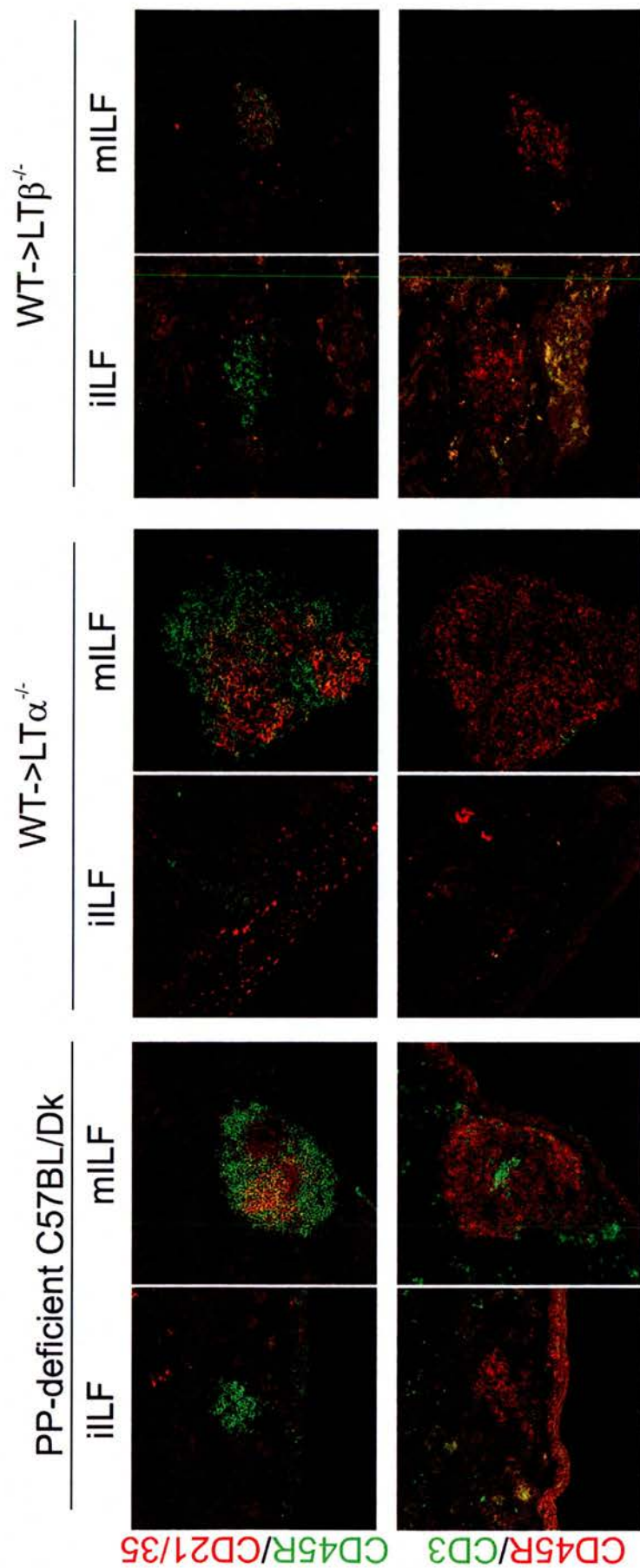
**Figure 3.9- Enumeration of isolated lymphoid follicles (ILFs) and follicular dendritic cells (FDCs) in the small intestine of WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice 105 days after reconstitution with wild-type (WT) bone marrow.** **a.** Similar numbers of ILFs were found in WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice. **b.** WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  mice were found to have a significantly higher percentage of mature ILFs (mILFs) when compared to WT->WT mice. **c.** FDC area per mILF was taken as a measure of mILF size. WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice were found to have significantly larger mILFs when compared to WT->WT mice. **d.** The total area covered by FDCs in all the mILFs in WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice was equivalent to the total area covered by FDCs in all the PPs and mILFs in WT->WT mice. Each bar represents the mean  $\pm$  S.E.M for each group of mice ( $n = 4$  for each group). \* indicates a  $p$  value of  $\leq 0.05$  compared to the WT->WT value. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are WT mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice respectively, reconstituted with WT bone marrow.



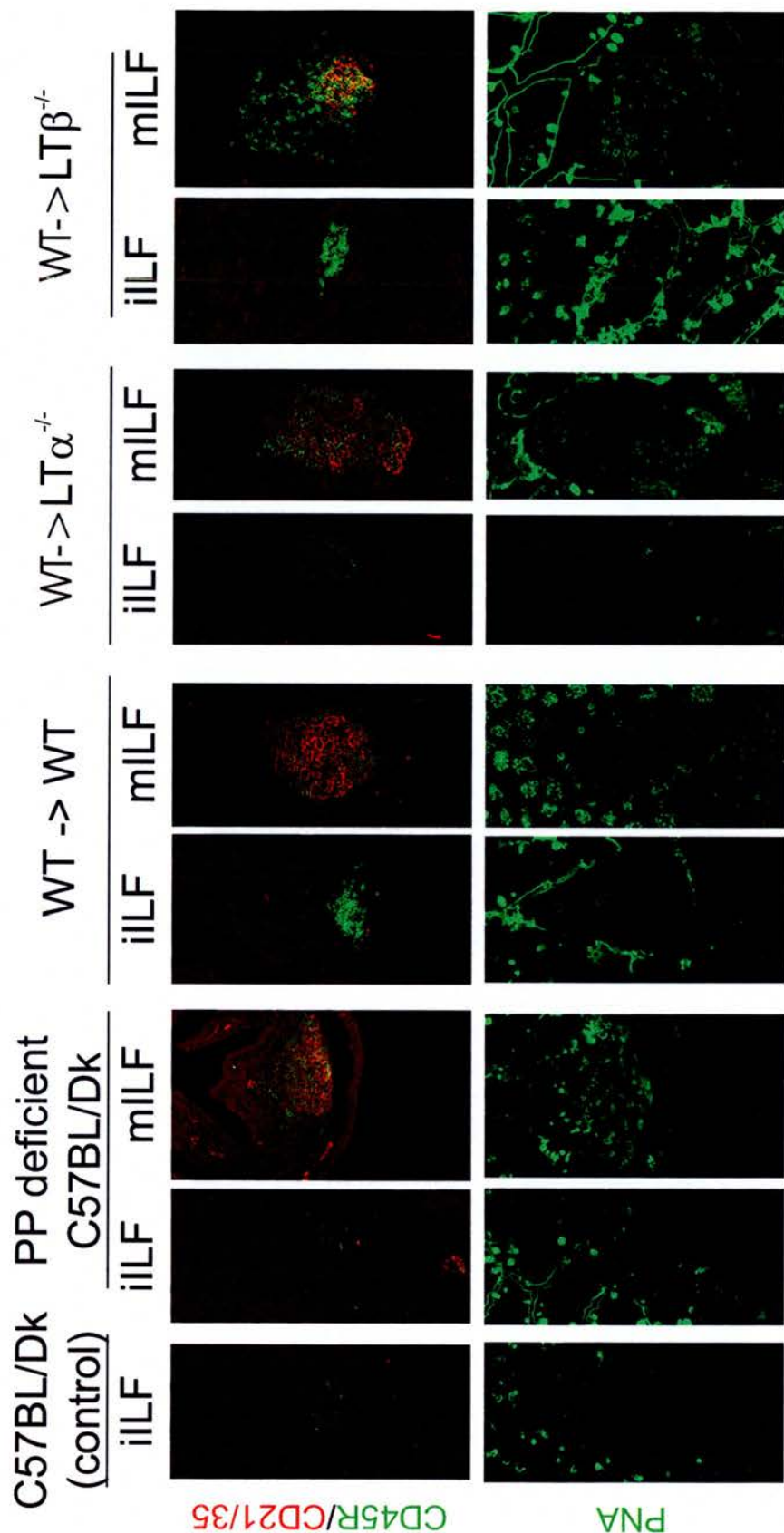
deficiency in terms of the total area covered by FDCs in the small intestine.

### **3.3.6 mILFs resemble fully functional lymphoid tissue**

Further immunohistochemical analysis revealed the presence of CD3-positive T lymphocytes within both mILF and iILFs of C57BL/Dk (control) mice, PP-deficient C57BL/Dk mice, WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice although these cells appeared to be more frequent in mILFs (Fig 3.10). Germinal centre B lymphocytes (PNA-positive cells) were detected in mILFs in all five groups of mice and were rarely detected in iILFs (Fig 3.11). Studies suggest that 97.2 % of follicle associated epithelial (FAE) M cells stain with UEA-1, which detects  $\alpha$ -L-fucose expressing cells (Clark *et al.*, 1993). In comparison, only 1 % of enterocytes are UEA-1 positive and goblet cells do not stain with UEA-1 (Fig 3.12, white arrowheads; Clark *et al.*, 1993). A recent study has also revealed the presence of UEA-1 positive M cells in the epithelia of villi in the murine small intestine (Jang *et al.*, 2004). UEA-1 positive M cells were detected in the FAE of PPs from C57BL/Dk (control) mice and WT->WT mice as well as in the epithelia of the ILFs and villi in the intestines of C57BL/Dk (control) mice, PP-deficient C57BL/Dk mice, WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice (Fig 3.12, black arrows). Lyve-1 is a receptor for hyaluronan, and is expressed predominantly on lymph vessels such that anti-Lyve-1 immunostaining in the intestine is lymphatic vessel specific (Banerji *et al.*, 1999). Lyve-1 positive cells were detected within villi and ILFs of PP-deficient C57BL/Dk mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice (Fig 3.13) suggesting that ILFs, like PPs and lymph nodes are connected to the lymphatic vasculature.

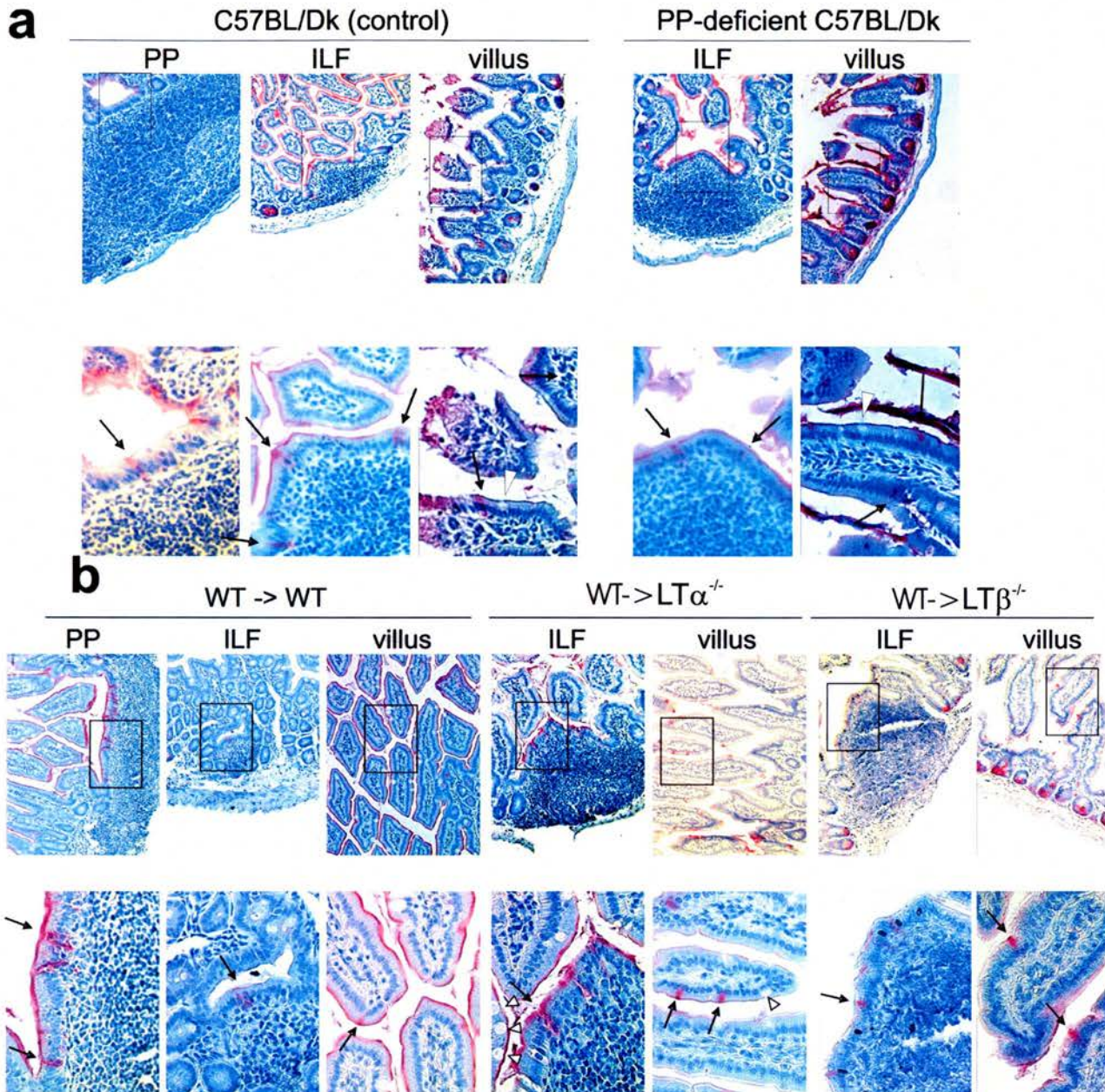


**Figure 3.10- Presence of T lymphocytes in isolated lymphoid follicles (ILFs).** Adjacent sections of small intestine from PP-deficient C57BL/Dk (left and 2<sup>nd</sup> columns), wild-type bone marrow reconstituted LT $\alpha^{-/-}$  mice (WT->LT $\alpha^{-/-}$  mice; 3<sup>rd</sup> and 4<sup>th</sup> columns) and WT->LT $\beta^{-/-}$  mice (5<sup>th</sup> and right columns) were stained with CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green, top row; red, lower row) and CD21/35 specific monoclonal antiserum to detect follicular dendritic cells (FDCs; red; top row), and CD3-specific monoclonal antiserum to detect T lymphocytes (green; lower row). T lymphocytes were found in mature ILFs (mILFs; lower 2<sup>nd</sup>, 4<sup>th</sup> and right columns) whereas few or no T lymphocytes were found in immature ILFs (iILFs; lower left, 3<sup>rd</sup> and 5<sup>th</sup> columns). Original magnification X200.



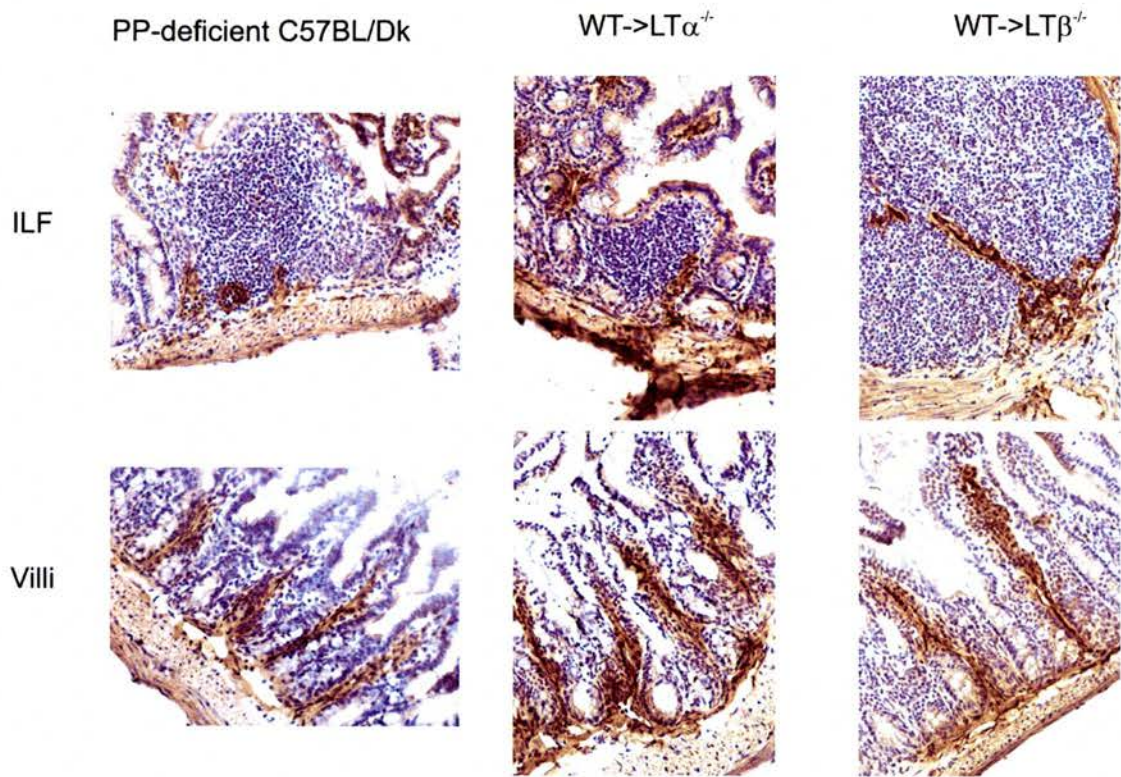
**Figure 3.11- Presence of germinal centre B lymphocytes in isolated lymphoid follicles (ILFs).** Adjacent sections of small intestine were stained with CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green; top row) and CD21/35-specific monoclonal antiserum to detect follicular dendritic cells (FDCs; red; top row), and peanut agglutinin (PNA) to detect germinal centre B lymphocytes (green; lower row). PNA positive germinal centre B lymphocytes were detected in mature ILFs (mILFs; lower 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup> and right columns) whereas none were detected in immature ILFs (iILFs; lower left 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> columns). Original magnification X200.





**Figure 3.12- M cells are present in the epithelium overlying Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and villi.** Sections of small intestine from **a.** C57BL/Dk (control) mice and PP-deficient C57BL/Dk mice and **b.** wild-type (WT) mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice reconstituted with WT bone marrow (WT→WT mice, WT→LT $\alpha^{-/-}$  mice and WT→LT $\beta^{-/-}$  mice respectively) were stained with *Ulex europaeus* agglutinin-1 (UEA-1) to detect M cells (red) and counterstained with haematoxylin (blue). Goblet cells (white arrowheads) were left unstained by UEA-1. Original magnification X200 (top row) or X600 (lower row).



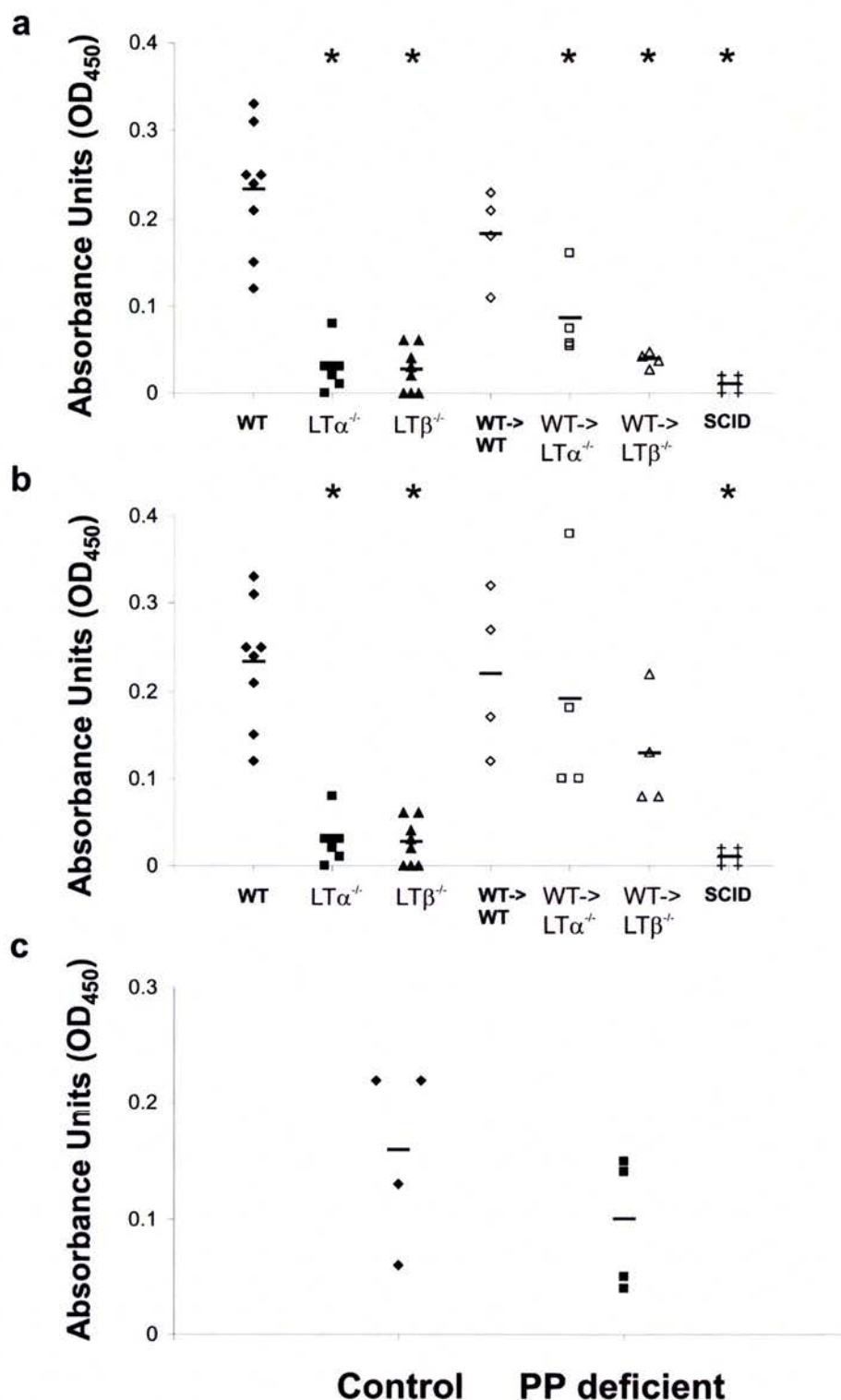


**Figure 3.13- Lymphatic vessels were found within isolated lymphoid follicles (ILFs; top row) and villi (lower row) in the small intestine of PP-deficient C57BL/Dk mice, wild-type bone marrow reconstituted LT $\alpha^{-/-}$  mice (WT->LT $\alpha^{-/-}$  mice) and WT->LT $\beta^{-/-}$  mice. Sections of small intestine were stained with monoclonal antiserum Lyve-1 to detect lymphatic vessels (brown) and counterstained with haematoxylin (blue). Original magnification X200.**

### **3.3.7 Enhanced intestinal IgA synthesis in the presence of mILFs**

The results in this chapter and the work of others (Hamada *et al.*, 2002; Lorenz *et al.*, 2003) have shown that mILFs resemble fully functional lymphoid tissue. To investigate whether mILFs could facilitate the synthesis of IgA in the intestine levels of faecal IgA were measured. Whereas IgA was detected in the faeces of WT->WT and C57BL/Dk (control) mice, no faecal IgA was detected in faeces from SCID mice (Fig 3.14). Likewise, due to an absence of PPs and ILFs in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice, no IgA was detected in the faeces of these mice. The level of IgA was also low in WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice 35 days after reconstitution with WT bone marrow and not significantly different from their unreconstituted counterparts ( $p \leq 0.491$ ,  $n = 4$  and  $p \leq 0.392$ ,  $n = 4$ , respectively) consistent with mILFs being absent from the intestines of these mice at this time point (Fig 3.6). Interestingly, only low levels of IgA were detected in WT-> $LT\beta^{-/-}$  mice despite the presence of FDC-containing MLNs in these mice at this point (Fig 4.8). In contrast, 105 days after bone marrow reconstitution when the formation of mILFs had been induced in WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice, the levels of faecal IgA were significantly higher than those detected in their unreconstituted counterparts ( $p \leq 0.010$ ,  $n = 4$  and  $p \leq 0.030$ ,  $n = 4$ , respectively) and similar to those detected in the faeces of WT mice ( $p \leq 0.722$ ,  $n = 4$  and  $p \leq 0.152$ ,  $n = 4$  respectively; Figure 3.14). Similarly, in PP-deficient C57BL/Dk mice which had no PPs but increased numbers of mILFs in their intestines due to *in utero*  $LT\beta$ R signalling blockade, similar levels of faecal IgA were found when compared to C57BL/Dk (control) mice ( $p \leq 0.245$ ,  $n = 4$ ; Fig 3.14). Taken together these results suggest that in the absence of PPs, mILFs are important





**Figure 3.14- Faecal IgA levels.** Each point represents the mean optical density (OD) at 450 nm for triplicate samples from individual mice. — represents mean OD at 450 nm for each experimental group of between 4 and 8 mice. Wild-type (WT) mice were found to have similar levels of IgA before (closed diamonds, **a** and **b**) and after (open diamonds, **a** and **b**) reconstitution with WT bone marrow. Faecal IgA levels in both LTα<sup>-/-</sup> mice (squares) and LTβ<sup>-/-</sup> mice (triangles) mice were extremely low before reconstitution with WT bone marrow (closed symbols, **a** and **b**) and at 35 days after reconstitution (open symbols **a**) but increased significantly by 105 days after reconstitution (open symbols, **b**). **c.** C57BL/Dk (control) mice (diamonds) and PP-deficient C57BL/Dk mice (squares) were found to have equivalent levels of faecal IgA. SCID/*Prnp*<sup>+/+</sup> mice (crosses) were used as negative controls. \* indicates a *p* value of ≤ 0.05 compared to the value from WT mice.

sites of intestinal IgA production and that MLNs do not contribute to intestinal IgA.

### **3.3.8 Effect of age on ILF development**

Next, the effect of host age on ILF development was investigated. C57BL/Dk mice were aged to 900 days and compared with younger mice aged ~ 300 days old. In contrast to younger mice where both iILFs and mILFs were detected, no mILFs and significantly fewer iILFs were detected in old mice ( $p \leq 0.020$ ; Table 3.1). The number of PPs was similar in both old and young mice. However, in old mice, FDC networks were undetectable by CR1/2 staining in 3 out of 11 PPs analysed, and showed only weak staining in a further 7 PPs. In only one PP from an old mouse was CR1/2 staining comparable to that of young mice (Fig 3.15). This suggests that the immunosenescence observed in PPs with increased age also occurs in mILFs.

	Number of PPs	Number of iILFs	Number of mILFs
Young	2 ±0.5	8 ±1.4	2 ±0.6
Aged	3 ±0.9†	2 ±1.0*	0

**Table 3.1- Lymphoid tissue in the small intestine of young and aged mice.** Small intestine sections of young C57BL/Dk mice were compared to those of aged (~900 days old) mice. Numbers represent the mean figure for each group of mice ± S.E.M. PP = Peyer's patch; iILF = immature isolated lymphoid follicle; mILF = mature isolated lymphoid follicle.

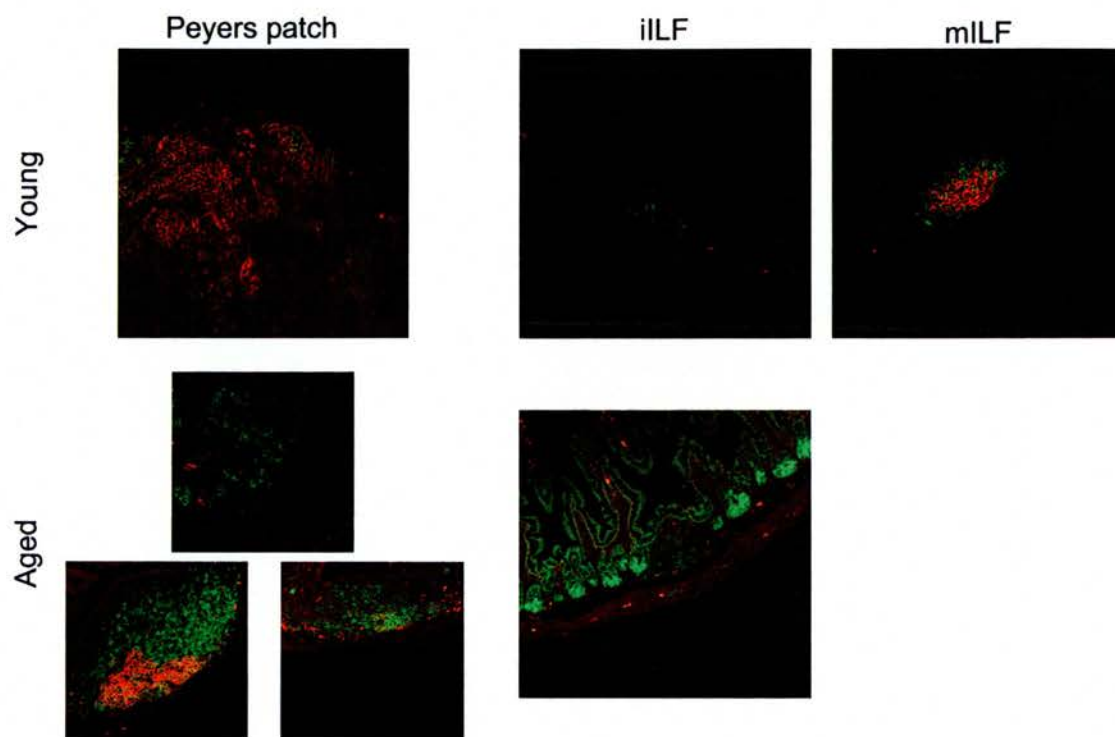
\* denotes  $p \leq 0.05$  compared to young

† 1/11 PPs contained both FDCs and B cells

7/11 PPs contained strong B220 staining and weak FDC staining

3/11 PPs contained weak B220 staining and no FDCs





**Figure 3.15- Lymphoid tissue architecture in the small intestine of young (~300 day old) and aged (~900 day old) mice.** Small intestine sections were stained with the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green) and 7G6 monoclonal antiserum to detect CD21/35 expressing follicular dendritic cells (FDCs; red). Tissues from young mice (top row) showed the presence of B lymphocyte and FDC containing Peyer's patches (PPs; left column), B lymphocyte containing immature isolated lymphoid follicles (iILFs; middle column) and B lymphocyte and FDC containing mature isolated follicles (mILFs; right column). Tissues from aged mice (lower row) showed the presence of PPs (right column) and iILFs (middle column). No mILFs were detectable in aged mice. Original magnification X200.

### **3.4 Discussion**

In this chapter ILFs were investigated in the small intestines of mice that lack PPs either due to *in utero* treatment with LT $\beta$ R-Ig or due to genetic deficiency of either LT $\alpha$  or LT $\beta$ . In both cases, FDC presence was found to be a consistent marker of ILF maturity. Increased numbers of larger mILFs were found in mice lacking PPs compared to relevant control mice, and these mILFs were found to adequately compensate for the lack of PPs such that the total area covered by FDCs in the intestines of all groups of mice investigated were equivalent. mILFs were also found to contain T lymphocytes, germinal centre B lymphocytes, lymphatic vessels and the FAE overlying the mILFs contained M cells. The detection of IgA in the intestines of mice lacking PPs or both PPs and MLNs correlated with the presence of mILFs. Taken together these data clearly demonstrate that mILFs are fully functional gut-associated lymphoid tissues.

The formation of PPs is critically dependent on LT $\alpha_1\beta_2$  signalling through LT $\beta$ R during gestation (Banks *et al.*, 1995; Honda *et al.*, 2001; Koni *et al.*, 1997; Rennert *et al.*, 1996; Togni *et al.*, 1994; Yoshida *et al.*, 1999). As a consequence, PPs do not develop in mice in which this signalling is interrupted during gestation, either by treatment with LT $\beta$ R-Ig (Rennert *et al.*, 1996) or due to genetic deficiency of either LT $\alpha$  (Togni *et al.*, 1994) or LT $\beta$  (Koni *et al.*, 1997). Unlike PPs, the development of ILFs, while still being critically dependent on LT signalling, occurs postnatally (Hamada *et al.*, 2002; Lorenz *et al.*, 2003). ILFs are, therefore, present in *in utero* LT $\beta$ R-Ig treated mice as the LT $\beta$ R signalling blockade is temporary and LT signalling is restored by the time ILFs form (from approximately 25 days after birth;

Hamada *et al.*, 2002). However, ILFs are not present in mice genetically deficient in either  $LT\alpha^{-/-}$  or  $LT\beta^{-/-}$  as the required signalling can never take place in these animals.

The cells providing the source of LT for the generation of PP anlage and ILF anlage are phenotypically distinct. PP formation is critically dependent on  $LT\alpha_1\beta_2$ -expressing  $CD4^+CD3^-$  lymphoid tissue initiating cells of bone marrow origin interacting with  $LT\beta R$ -expressing stromal cells of mesenchymal origin (Adachi *et al.*, 1998; Finke *et al.*, 2002; Honda *et al.*, 2001; Yoshida *et al.*, 2002). The formation of ILFs, on the other hand, has been found to be critically dependent on  $LT\alpha_1\beta_2$ -expressing NK cells, B lymphocytes or T lymphocytes interacting with  $LT\beta R$ -expressing stromal cells (Lorenz *et al.*, 2003; McDonald *et al.*, 2005). Thus, it follows that the reconstitution of either  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice with WT bone marrow results in the restoration of ILF development without causing PPs to be formed. Previous work has shown that mILFs do not form in  $LT\alpha^{-/-}$  mice reconstituted with LT-sufficient NK cells and T lymphocytes (McDonald *et al.*, 2005). However, the formation of mILFs does occur in both  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice reconstituted with LT-sufficient NK cells and B lymphocytes and T lymphocytes (Figs 3.5 & 3.6; Lorenz *et al.*, 2003; McDonald *et al.*, 2005). Taken together these results show the necessity of LT-sufficient B lymphocytes for the formation of mILFs. The source of  $LT\alpha_1\beta_2$  for the formation of iILFs can be either B-lymphocytes, T-lymphocytes or NK cells as iILFs were found in the intestines of mice reconstituted with LT-sufficient T-lymphocytes and NK cells, or NK cells alone (Lorenz *et al.*, 2003; McDonald *et al.*, 2005).



The work of others demonstrated the presence of ILFs in  $LT\alpha^{-/-}$  mice 12 weeks after reconstitution with WT bone marrow (Lorenz *et al.*, 2003). Consistent with this, data presented here describes the absence of ILFs in  $LT\alpha^{-/-}$  mice 5 weeks after reconstitution with WT bone marrow, and their presence by 15 weeks after bone marrow reconstitution. In addition, data presented here also shows that the formation of ILFs in  $WT \rightarrow LT\beta^{-/-}$  mice occurs similarly to  $WT \rightarrow LT\alpha^{-/-}$  mice which is confirming that ILF formation is dependent upon a signalling pathway in which both  $LT\alpha$  and  $LT\beta$  play a part.

Immature ILFs have previously been defined as loose clusters of B220-positive cells located at the base of a villus, and mILFs as well organised nodular structures the width of one or more villi (Lorenz *et al.*, 2003). In this chapter, the presence of FDCs was demonstrated in mILFs but not in iILFs. Thus the presence of FDCs can also be used as criterion for determining the maturity of ILFs. In the morphometric analysis described in this chapter anti-CR1/CR2 antiserum was used to detect FDCs as they characteristically express these receptors at high levels. However, in the mouse CR1 and CR2 are also expressed on B lymphocytes, but at lower levels than on FDCs. Thus calculations were based solely on  $CR1/CR2^{+} CD45R^{-}$  cells. The presence of FDCs in mILFs was confirmed by the colocalisation of immunostaining for complement receptors CR1 and CR2. Co-incident immunostaining between CR1 and CR2 and complement components C3 and C4 demonstrated that the FDCs in mILFs were also exhibiting the function of immune-complex trapping.

The number of ILFs in mice lacking PPs due to *in utero* LT $\beta$ R-Ig treatment was found to be increased when compared to the C57BL/Dk (control) mice. However, the number of ILFs in WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice was similar to the number in WT->WT control mice. The mILFs of WT->LT $\alpha^{-/-}$  mice were found to be significantly larger than the mILFs of the LT $\beta$ R-Ig treated mice. These variations might be explained by the different strains of mice that were used as there are also significant differences in the number and size of ILFs between C57BL/Dk mice and WT (C57BL/6) mice, and the work of others has shown similar differences in ILF number between different mouse strains (Hamada *et al.*, 2002; Lorenz & Newberry, 2004). In both cases, the absence of PPs induced a significant increase in the size and maturity of ILFs when compared to the respective controls. The increase in size and maturity of ILFs resulted in the total area occupied by FDCs in the intestines of the mice being similar to the respective controls and to each other. This showed that although the distribution of FDCs within the intestines of these distinct groups of mice was different, the overall area occupied by FDCs was the same, suggesting that the reason why ILFs form in both cases is to make sure the intestines are not unprotected immunologically in the absence of PP function.

Consistent with previous reports (Hamada *et al.*, 2002; Lorenz *et al.*, 2003), mILFs were found to contain T lymphocytes and germinal centre B lymphocytes. Previous work using electron microscopy has also shown the presence of M cells in the FAE overlying ILFs (Hamada *et al.*, 2002; Lorenz *et al.*, 2003). Consistent with this, the presence of UEA-1 positive M cells was detected in FAE overlying ILFs and also in villi in the work described here. The pathogenic bacterium *Yersinia enterocolitica*

exploits M cells to invade the host (Clark *et al.*, 1998; Dube *et al.*, 2003; Isberg & Leong, 1990). Thus the presence of *Y. enterocolitica* within the GALT can be used to demonstrate M cell-mediated transcytosis. The work of others has shown that *Y. enterocolitica* can infect mILFs demonstrating that the M cells overlying these structures are functional (Lorenz & Newberry, 2004). Experiments described here also show that ILFs are connected to the lymphatic vasculature by the presence of Lyve-1 positive vessels in ILFs. PPs have efferent lymphatics but no afferent lymphatics and so represent the beginning of a lymphatic vessel. Lymph nodes, in contrast, have both afferent and efferent lymphatic vessels. It is likely that, as ILFs are more similar to PPs than they are to lymph nodes, they would also represent the beginning of a lymphatic vessel. Taken together, these results show that mILFs resemble fully functional lymphoid tissue.

IgA was detected in the intestines of mice containing mILFs and lacking PPs and MLNs. IgA was absent from mice lacking PPs, MLNs and mILFs despite the presence of FDCs in remaining lymphoid tissues in these mice. This suggested that IgA synthesis in the intestines of mice with no PPs or MLNs was dependent on the presence of mILFs. These observations are consistent with the work of others which has shown that after infection with *Salmonella typhimurium*, specific IgA was present in intestines of mice which had mILFs, but lacked PPs or MLNs (Lorenz & Newberry, 2004). Likewise, cells isolated from the mILFs of sheep red blood cell (SRBC) immunised mice have been shown to be capable of making SRBC-specific IgA upon restimulation (Lorenz & Newberry, 2004). Collectively, these data suggest



that mILFs are capable of producing IgA, and, therefore, display similar functionality to other gut-associated lymphoid tissues.

Most mutations that would give rise to PP deficiency will also result in general immunodeficiency and, therefore, increased susceptibility to many infectious diseases. For example, mice lacking various components of the  $LT\alpha_1\beta_2$  signalling pathway have been shown to control intracellular bacterial (Bopst *et al.*, 2001; Ehlers *et al.*, 2003; Engwerda *et al.*, 2004; Fontan *et al.*, 2001; Jacobs *et al.*, 2000; Trueb *et al.*, 1995; Wilhelm *et al.*, 2002), protozoan (Engwerda *et al.*, 2004; Wilhelm *et al.*, 2002), viral (Banks *et al.*, 2005; Benedict *et al.*, 2001; Berger *et al.*, 1999; Kamaraguru *et al.*, 2001; Lund *et al.*, 2002; Suresh *et al.*, 2002) and metastatic (Dobrzanski *et al.*, 2004; Ito *et al.*, 1999; Smyth *et al.*, 1999) disease less well than immunocompetent counterparts. A naturally occurring PP deficient mouse is, therefore, unlikely to survive long enough to be able to reproduce. Thus the chances of a compensatory mechanism for PP deficiency evolving on this basis are not high. The formation of mILFs has been postulated to occur when factors secreted by PPs are absent (Lorenz *et al.*, 2003). The precise identity of these stimuli is unknown. As PP size decreases with age (Fujihashi & McGhee, 2004), the induction of ILF maturation might provide a mechanism to compensate for PP decline. If this were true, it could be postulated that the intestines of old mice may contain more mILFs than young counterparts. However, when the intestines of old mice were investigated no mILFs could be detected at all, suggesting that ILFs are affected by age in a similar way to PPs.

This leaves the reason for the evolution of the formation of ILFs open to question. One possible explanation is that the mechanism of formation of ILFs occurred earlier on the evolutionary timescale and has since been superceded by the development of PPs (Hamada *et al.*, 2002). In event of disruption to the mechanism of PP development, however, the mechanism for the formation of ILFs is still utilisable to prevent the intestines being unprotected immunologically. These ideas have yet to be investigated fully.

In conclusion, data presented here, consistent with the data of others, showed that the absence of PPs induced the development of an increased number of mILFs in the intestines. In this study, FDCs were found to be present in mILFs, but not in iILFs and the total area covered by FDCs in the intestine was found to be equivalent between all three types of PP deficient mice studied and their relevant controls. mILFs were found to both resemble and function as fully differentiated lymphoid tissue. As FDCs are important for the peripheral pathogenesis of TSEs these results would suggest that mILFs are likely to be an important site of TSE agent neuroinvasion from the intestine.

# 4

## Role of gut-associated lymphoid tissue in oral scrapie pathogenesis

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## **4.1 Abstract**

Mice lacking specific combinations of lymphoid tissues were inoculated orally with the ME7 scrapie agent to investigate the role of gut-associated lymphoid tissues (GALT) in scrapie agent neuroinvasion from the intestine. Signalling via the lymphotoxin (LT)  $\beta$  receptor during gestation is crucial for the formation of Peyer's patches (PPs) and other secondary lymphoid tissues;  $LT\beta^{-/-}$  mice lack PPs and isolated lymphoid follicles (ILFs) and  $LT\alpha^{-/-}$  mice lack PPs, ILFs and mesenteric lymph nodes (MLNs). In addition follicular dendritic cells (FDCs) also require LT-signalling to maintain their differentiated state; therefore, both  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice have no FDCs. In the absence of FDCs neither  $LT\alpha^{-/-}$  mice nor  $LT\beta^{-/-}$  mice are susceptible to oral infection with scrapie as FDCs are important for scrapie agent neuroinvasion after oral inoculation. Reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with wild-type (WT) bone marrow restores FDCs and lymphoid tissue microarchitecture, but does not initiate the formation of the missing PPs or MLNs. After reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow none of the recipient mice succumbed to scrapie, despite the presence of FDCs in the remaining lymphoid tissues. Thus in the absence of PPs and ILFs in the intestine, neuroinvasion is blocked. The MLNs appear not to be crucial for neuroinvasion from the intestines as FDC containing MLNs were present in WT bone marrow reconstituted  $LT\beta^{-/-}$  mice. Progeny mice treated *in utero* with  $LT\beta$ R-Ig lack PPs, but develop increased numbers of FDC-containing mature ILFs (mILFs) in their intestines. After oral inoculation with scrapie, PrP<sup>d</sup> accumulated on the FDCs in mILFs and both PP-deficient and immunocompetent control mice developed disease at the same time following oral inoculation. Taken together these results show that

after oral inoculation with the scrapie agent, efficient neuroinvasion occurs directly from FDCs in lymphoid tissue within the intestine such as PPs and mILFs. Although the scrapie agent accumulates in the MLNs shortly after oral inoculation, these tissues are not critical for efficient neuroinvasion.

## **4.2 Introduction**

Many natural TSE infections are thought to be acquired via the oral route. For example, the spread of BSE amongst UK cattle was most likely caused by the use of BSE-contaminated feed (Wilesmith *et al.*, 1991). The subsequent emergence of vCJD in humans is thought to be principally due to the consumption of BSE-infected meat as BSE and vCJD have been shown to be caused by the same strain of TSE agent (Bruce *et al.*, 1997; Hill *et al.*, 1997). Although the precise route of infection for natural scrapie remains unclear, the detection of disease specific PrP in the lymphoid tissues draining the intestines (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2000) prior to detection within other lymphoid tissues or the central nervous system (CNS; Keulen *et al.*, 1999) implies that this disease may also be acquired orally. In addition, both chronic wasting disease (CWD) in mule deer and elk (Williams & Young, 1980) and transmissible mink encephalopathy (TME; Hartsough & Berger, 1965) are most likely transmitted via the oral route.

The involvement of lymphoid tissues in scrapie agent neuroinvasion was first demonstrated in 1967 when it was reported that agent infectivity accumulates to high levels in the spleen and peripheral lymph nodes before it is detected in the CNS in experimentally infected mice (Eklund *et al.*, 1967). The precise mechanism through which the agent reaches the CNS is unclear, but *in vitro* and *in vivo* studies in rodents and sheep suggest that the scrapie agent may be transported from the intestinal lumen to the gut-associated lymphoid tissues (GALT) by dendritic cells (Huang *et al.*, 2002; Kaneider *et al.*, 2003), macrophages (Beringue *et al.*, 2000; Carp & Callaghan, 1982; Jeffrey *et al.*, 2000) or M cells (Beekes & McBride, 2000; Heggebo *et al.*, 2002;



Heppner *et al.*, 2001). Once in the lymphoid tissues the scrapie agent accumulates on follicular dendritic cells (FDCs; Jeffrey *et al.*, 2000; McBride *et al.*, 1992). Scrapie agent accumulation on FDCs has been found to be of crucial importance for neuroinvasion as treatment of adult mice with LT $\beta$ R-Ig to temporarily de-differentiate FDCs 3 days prior to oral inoculation blocked the transmission of the disease to the CNS (Mabbott *et al.*, 2003). In contrast, mice treated with LT $\beta$ R-Ig 14 days after oral inoculation were just as susceptible to scrapie as immunocompetent controls (Mabbott *et al.*, 2003) thus demonstrating that neuroinvasion following oral inoculation was rapid and most likely occurred directly from lymphoid tissue within the intestine.

The gut associated lymphoid tissue (GALT) comprises chiefly of appendix, tonsils, Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and isolated lymphoid follicles (ILFs). Although TSE agents accumulate in the GALT following oral exposure, which, if any, of these tissues are crucial for disease pathogenesis is unclear. It is plausible that one component of the GALT (e.g. PPs) is of paramount importance and the development of disease is prevented in its absence. It is also plausible that neuroinvasion occurs from multiple sites, such that lack of one type of lymphoid tissue (e.g. PPs) can be compensated for by the presence of another (e.g., MLNs). It is also plausible that the GALT is not critically required and that neuroinvasion can take place from other sites (e.g. spleen) in its total absence. To address this issue, mice lacking combinations of PPs, MLNs and ILFs were inoculated orally with ME7 scrapie to determine the contribution of each of these tissues to neuroinvasion from the intestine.

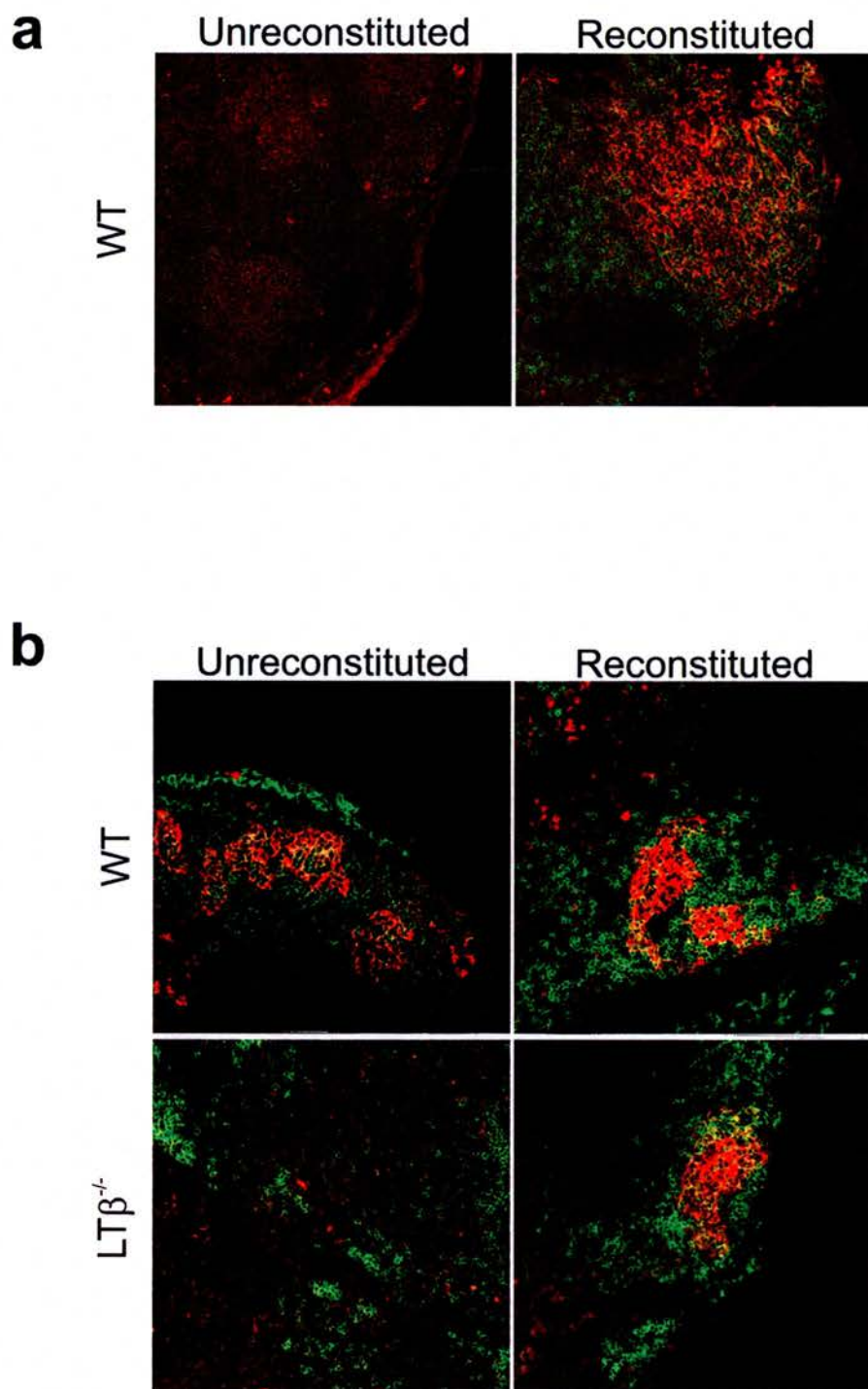
## **4.3 Results**

### **4.3.1 Lymphoid tissues of lymphotoxin $\alpha$ deficient ( $LT\alpha^{-/-}$ ) mice and lymphotoxin $\beta$ deficient ( $LT\beta^{-/-}$ ) mice lack FDCs**

First, FDC presence in the remaining lymphoid tissues of  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and wild-type (WT) immunocompetent mice was compared. Sections of spleen and MLN (where present) were stained with CD21/35 specific monoclonal antiserum 7G6 to detect CR1/CR2 expressing FDCs and CD45R-specific monoclonal antiserum B220 to detect B lymphocytes. As expected, large FDC networks were detected within the organised B lymphocyte follicles of PPs, MLNs and spleens from WT mice. In contrast, FDCs and distinguishable B lymphocyte follicles were not detected in the remaining lymphoid tissues from either  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice (Figs 4.1 & 4.2).

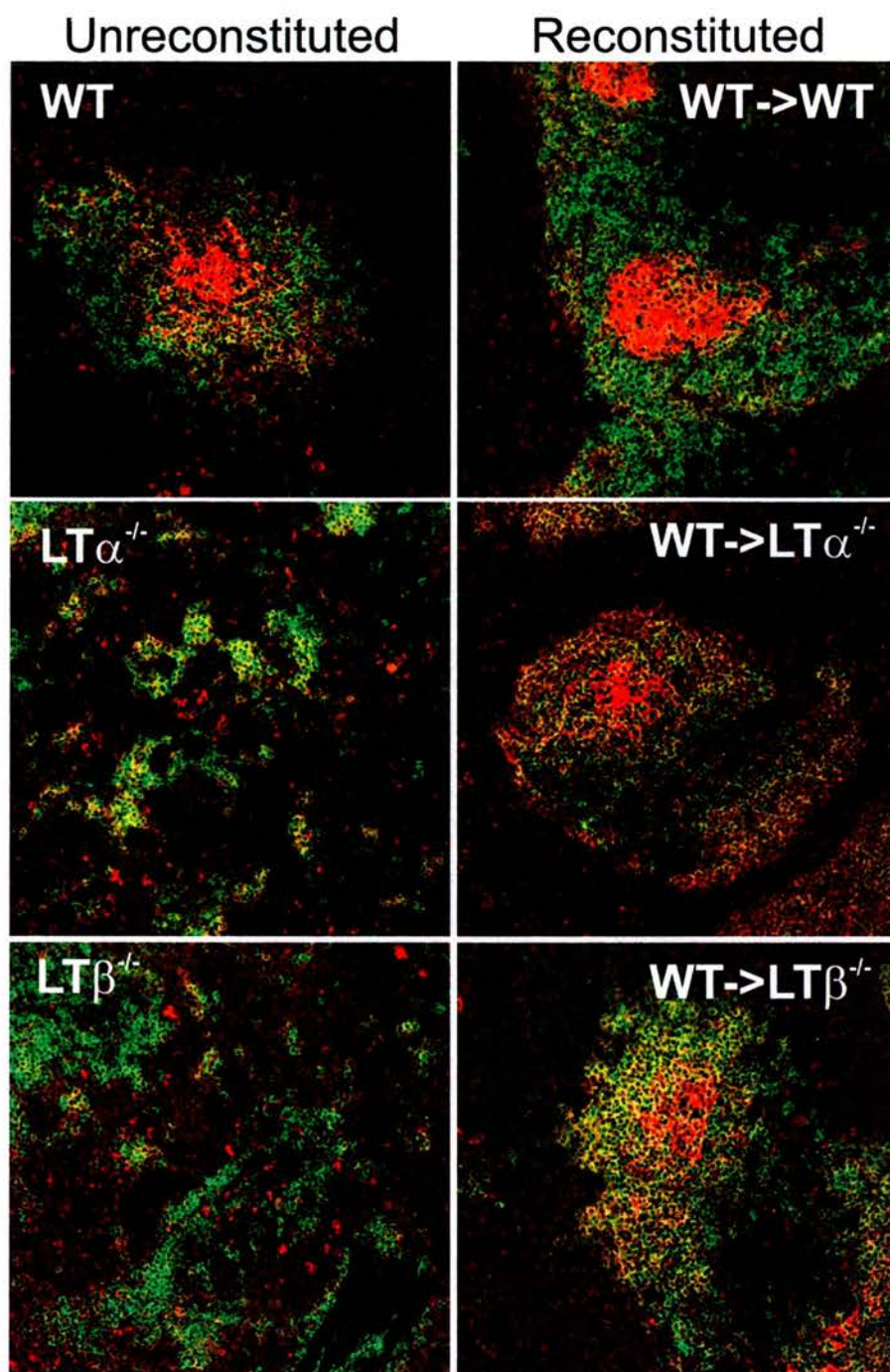
### **4.3.2 Effect of lymphotoxin (LT) deficiency on susceptibility to scrapie infection**

Next the potential effect LT deficiency may have on the pathogenesis of scrapie directly within the CNS was determined. After intra-cranial inoculation of WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with 20  $\mu$ l of a 1.0 % dilution of ME7 scrapie brain homogenate no significant differences in the incubation periods of disease were observed ( $171 \pm 4$  days,  $n = 6$ ;  $175 \pm 4$  days,  $p \leq 0.482$ ,  $n = 5$ ; and  $175 \pm 1$  days;  $p \leq 0.395$   $n = 5$  for WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice respectively; Table 4.1). These data show that genetic deficiency of either  $LT\alpha$  or  $LT\beta$  had no effect on the pathogenesis of ME7 scrapie directly within the CNS. Therefore, any effects observed in  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice after peripheral inoculation could be attributed to an altered efficiency of the translocation of the scrapie agent to the CNS.



**Figure 4.1- Effect of reconstitution with wild-type (WT) bone marrow on the microarchitecture of gut-associated lymphoid tissue.** Sections of **a)** Peyer's patches (PPs) from wild-type (WT) mice and **b)** mesenteric lymph nodes (MLNs) from WT mice and lymphotoxin  $\beta$  deficient ( $LT\beta^{-/-}$ ) mice were stained with the CD45R-specific antiserum B220 to detect B lymphocytes (green) and monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; red). PP and MLN tissues from WT mice (top rows) showed presence of FDCs and organised follicles in both unreconstituted mice and those which had been reconstituted with WT bone marrow. In unreconstituted  $LT\beta^{-/-}$  mice, MLN tissues showed a lack of FDCs and no distinct follicles (lower left). After reconstitution with WT bone marrow, FDCs and follicular organisation was restored in MLNs of  $LT\beta^{-/-}$  mice (lower right). Original magnification X200.





**Figure 4.2- Effect of reconstitution with wild-type (WT) bone marrow on the microarchitecture of spleens of WT mice, lymphotoxin  $\alpha$  deficient ( $LT\alpha^{-/-}$ ) mice and lymphotoxin  $\beta$  deficient ( $LT\beta^{-/-}$ ) mice.** Spleen sections were stained with the CD45R-specific antiserum B220 to detect B lymphocytes (green) and monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; red). Spleen tissues from WT mice (top row) showed presence of FDCs and organised follicles in both unreconstituted mice and mice which had been reconstituted with WT bone marrow (WT->WT mice). In unreconstituted  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice, spleen tissues showed a lack of FDCs and no distinct follicles (middle and lower left). After reconstitution with WT bone marrow, FDCs and follicular organisation was restored in spleens of both  $LT\alpha^{-/-}$  mice (WT-> $LT\alpha^{-/-}$  mice; middle right) and  $LT\beta^{-/-}$  mice (WT-> $LT\beta^{-/-}$  mice; lower right). Original magnification X200.

	Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.
WT	6/6	171 ± 4
LTα <sup>-/-</sup>	5/5	175 ± 4
LTβ <sup>-/-</sup>	5/5	175 ± 1

**Table 4.1- Effect of lymphotoxin (LT) deficiency on scrapie susceptibility after intra-cranial inoculation.**

1. Incidence = number of animals affected/ number of animals tested.  
 WT = wild-type mice; LTα<sup>-/-</sup> = lymphotoxin -α deficient mice; LTβ<sup>-/-</sup> = lymphotoxin -β deficient mice.

After oral inoculation, all WT mice succumbed to disease with a mean incubation period of  $311 \pm 3$  days ( $n = 20$ ) whereas all  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice remained free of the signs of scrapie until at least 545 days post inoculation (dpi; Table 4.2). In this thesis, disease-specific abnormal accumulations of PrP that are characteristically found only in TSE-affected tissues are termed  $PrP^d$ . Where proteinase K resistance of these accumulations is confirmed the term  $PrP^{Sc}$  is used (section 1.1.4). Characteristic spongiform pathology,  $PrP^d$  accumulation and reactive astrocytes were detected in the brains of all WT mice that developed clinical signs of scrapie as described in section 2.3.6 (clinically positive; Fig 4.3). The severity and distribution of the pathological targeting of vacuolation in the brains of WT mice was typical of ME7 scrapie (Fig 4.4). In contrast, no evidence of spongiform pathology,  $PrP^d$  accumulation or reactive astrocytes were detected in the brains of surviving  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice (Fig 4.3). Thus in the absence of FDCs and GALT, the neuroinvasion of the scrapie agent from the intestine was blocked.

### **4.3.3 Effect of lymphoid tissue and FDC deficiency on early and late $PrP^{Sc}$ accumulation in lymphoid tissues**

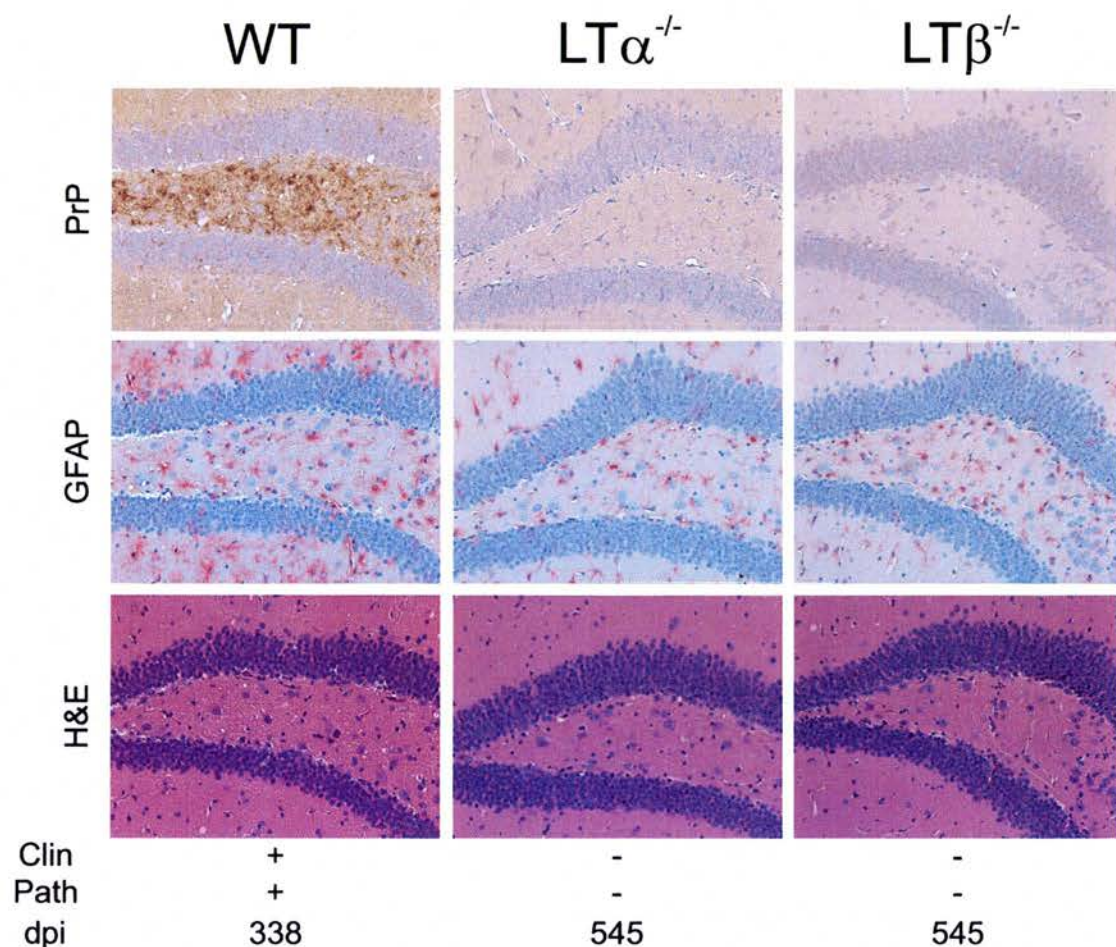
Within 70 days of oral inoculation with the ME7 scrapie agent high levels of the proteinase K (PK) resistant, disease specific isomer of PrP ( $PrP^{Sc}$ ) accumulate in the MLNs, but levels detected in the spleen are variable and often undetectable (Mabbott *et al.*, 2003). Consistent with previous reports, high levels of  $PrP^{Sc}$  were found in the MLNs and no  $PrP^{Sc}$  was found in the spleens of WT mice at this time point (Fig 4.5). In tissues of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice  $PrP^{Sc}$  was undetectable (Fig 4.5). At the terminal stage of disease high levels of  $PrP^{Sc}$  are found in both MLNs and spleens of



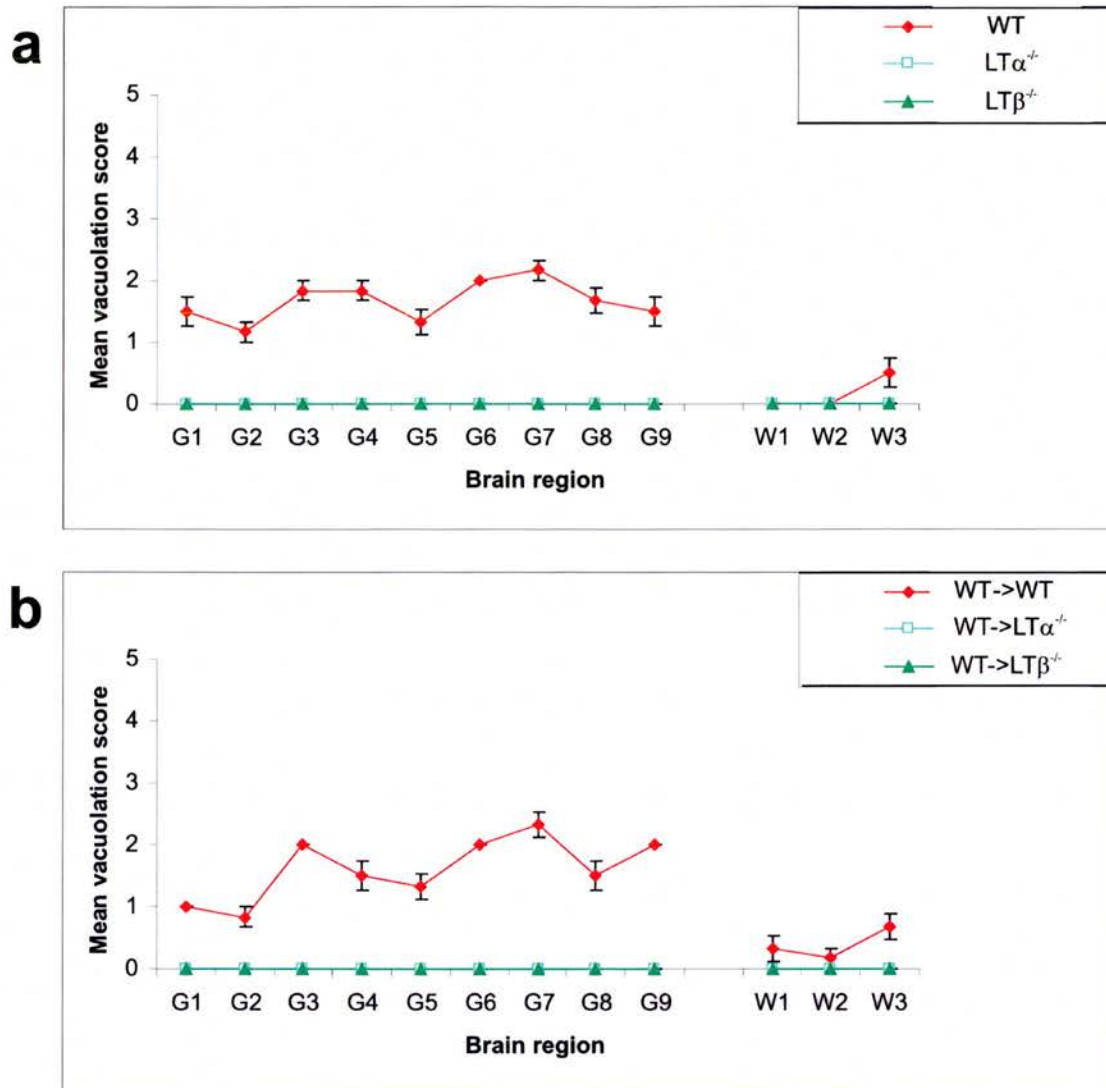
	Presence at time of inoculation					Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.	
	SPL		MLNs	PPs	mILFs			FDCs in remaining lymphoid tissues
WT	+	+	+	+	+/-	+	20/20	311 ± 3
LTα <sup>-/-</sup>	+		-	-	-	-	0/13	13x > 545
LTβ <sup>-/-</sup>	+	+		-	-	-	0/18	18x > 545

**Table 4.2 – Effect of lymphotoxin (LT) deficiency on scrapie susceptibility after oral inoculation.**

1. Incidence = number of animals affected/ number of animals tested. The notation nx > 545 means that mice were free of the signs of scrapie up to at least this time point after inoculation  
SPL = Spleen, MLNs = mesenteric lymph nodes, PPs = Peyer’s patches, mILFs = mature isolated lymphoid follicles, FDCs = follicular dendritic cells, WT = wild-type mice, LTα<sup>–/–</sup> = lymphotoxin –α deficient mice, LTβ<sup>–/–</sup> = lymphotoxin –β deficient mice  
+ = presence, – = absence (for SPL, MLNs, PPs and FDCs)  
+/– = presence of few (<10) mILFs compared to + = presence of many (>10) mILFs when sections of intestine were examined microscopically

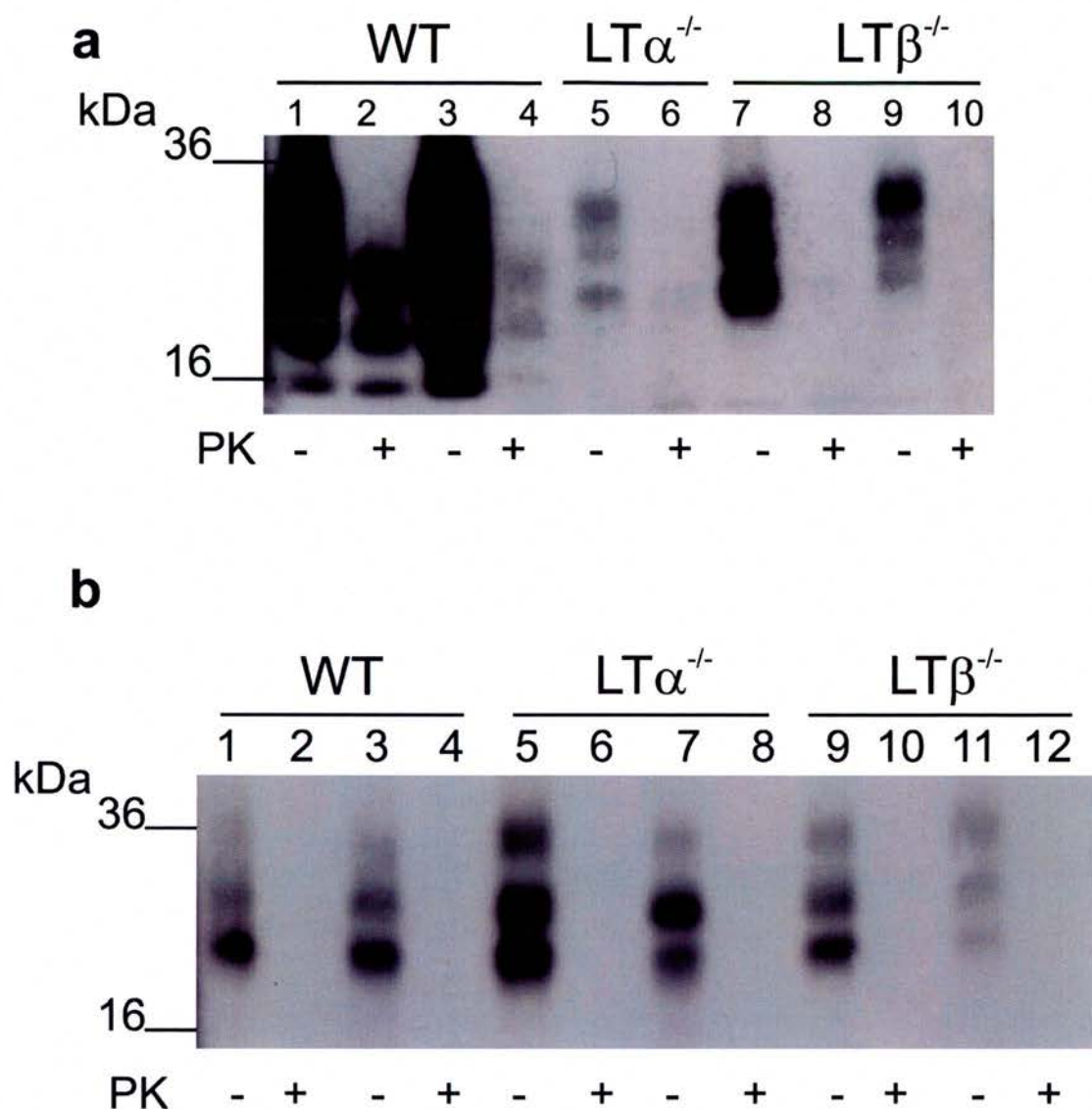


**Figure 4.3- Histological analysis of brain tissue from terminally scrapie affected mice or survivor mice.** Large disease specific PrP (PrP<sup>d</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. WT, wild-type mice; LT $\alpha^{-/-}$ , lymphotoxin  $\alpha$  deficient mice; LT $\beta^{-/-}$ , lymphotoxin- $\beta$  deficient mice; clin, clinical score, either positive (+) or negative (-); path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis.



**Figure 4.4- Pathological targeting of vacuolation in the brains of mice terminally affected by ME7 scrapie or survivor mice. a)** wild-type (WT) mice, lymphotoxin  $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice and lymphotoxin  $\beta$  deficient (LT $\beta^{-/-}$ ) mice were inoculated orally with ME7 scrapie. **b)** WT mice, LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice were reconstituted with WT bone marrow (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively) and inoculated orally with ME7 scrapie 35 days later. Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (G1-G9) or white matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of the superior peduncles; W3, cerebral peduncles. Each point represents mean vacuolation score  $\pm$  S.E.M. for groups of 6-9 mice.



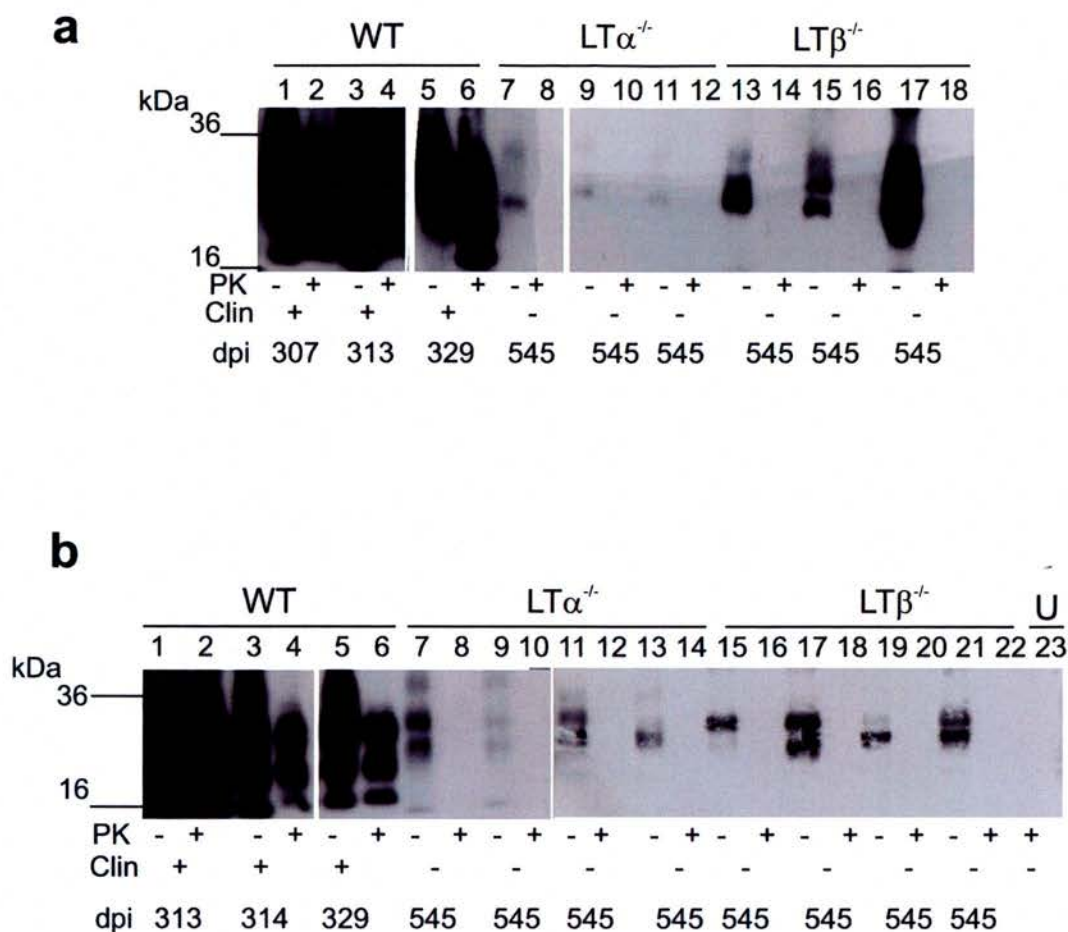


**Figure 4.5- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) Mesenteric lymph nodes (MLNs; or membrane where MLNs should have been in lymphotoxin- $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice) and (b) spleens of wild-type (WT) mice, LT $\alpha^{-/-}$  mice and lymphotoxin- $\beta$  deficient (LT $\beta^{-/-}$ ) mice 70 days after oral inoculation with ME7 scrapie. Proteinase K (PK) -resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in all the MLNs but not in the spleens of WT mice. No PrP<sup>Sc</sup> was detected in either MLNs or spleens from either LT $\alpha^{-/-}$  mice or LT $\beta^{-/-}$  mice. Samples were treated in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis.

immunocompetent mice (Mabbott *et al.*, 2003). Consistent with this, in the present study high levels of PrP<sup>Sc</sup> were detected in both the MLNs and spleens of WT mice (Fig 4.6). However, the accumulation of PrP<sup>Sc</sup> was blocked in lymphoid tissues of LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice taken from clinically negative animals that remained free from the signs of scrapie at 545 days after inoculation (end of the experiment; Fig 4.6).

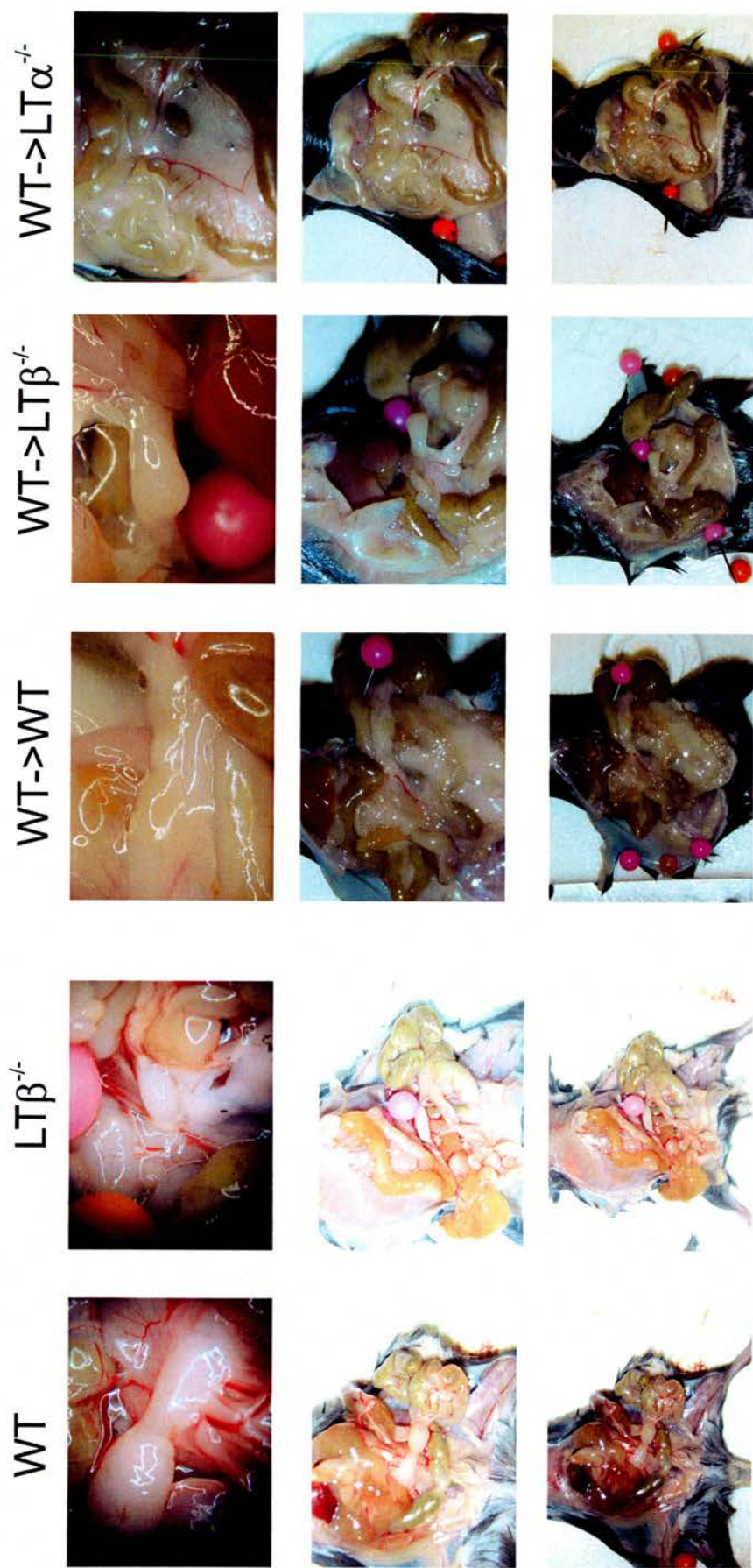
#### **4.3.4 Reconstitution of LT $\alpha$ <sup>-/-</sup> mice or LT $\beta$ <sup>-/-</sup> mice with WT bone marrow restores FDCs in remaining lymphoid tissues but does not initiate the formation of PPs or MLNs**

As well as there being a complete absence of FDCs from lymphoid tissues in LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice, PPs are also absent from both LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice and MLNs are absent from LT $\alpha$ <sup>-/-</sup> mice (Figs 3.5 & 4.7). Further experiments were therefore necessary to distinguish between the effect of FDC deficiency and lymphoid tissue deficiency on scrapie pathogenesis. Reconstitution of LT $\alpha$ <sup>-/-</sup> mice or LT $\beta$ <sup>-/-</sup> mice with WT bone marrow restores FDCs in remaining lymphoid tissues, but the absence of PPs and MLNs from LT $\alpha$ <sup>-/-</sup> mice and the absence of PPs from LT $\beta$ <sup>-/-</sup> mice is not recovered (Figs 3.5 & 4.7). WT mice, LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice were  $\gamma$ -irradiated and reconstituted with WT bone marrow (termed WT->WT, WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice respectively) and tissues collected 35 days later. Sections of PPs, MLNs (where present) and spleen were stained with CD21/35 specific antiserum to detect FDCs and anti-CD45R specific antiserum B220 to detect B lymphocytes. Immunohistochemical analysis revealed that FDCs were retained in



**Figure 4.6- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) mesenteric lymph nodes (MLNs; or membrane where MLNs should have been in lymphotoxin- $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice) and (b) spleens of clinically affected wild-type (WT) mice, or survivor LT $\alpha^{-/-}$  mice and lymphotoxin- $\beta$  deficient (LT $\beta^{-/-}$ ) mice after oral inoculation with ME7 scrapie. Proteinase K resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in the MLNs and the spleens of WT mice. No PrP<sup>Sc</sup> was detected in either MLNs or spleens from either LT $\alpha^{-/-}$  mice or LT $\beta^{-/-}$  mice, or from mice which were not inoculated with scrapie (U). Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score either positive (+) or negative (-); dpi, days post inoculation on which the tissues were taken for analysis.



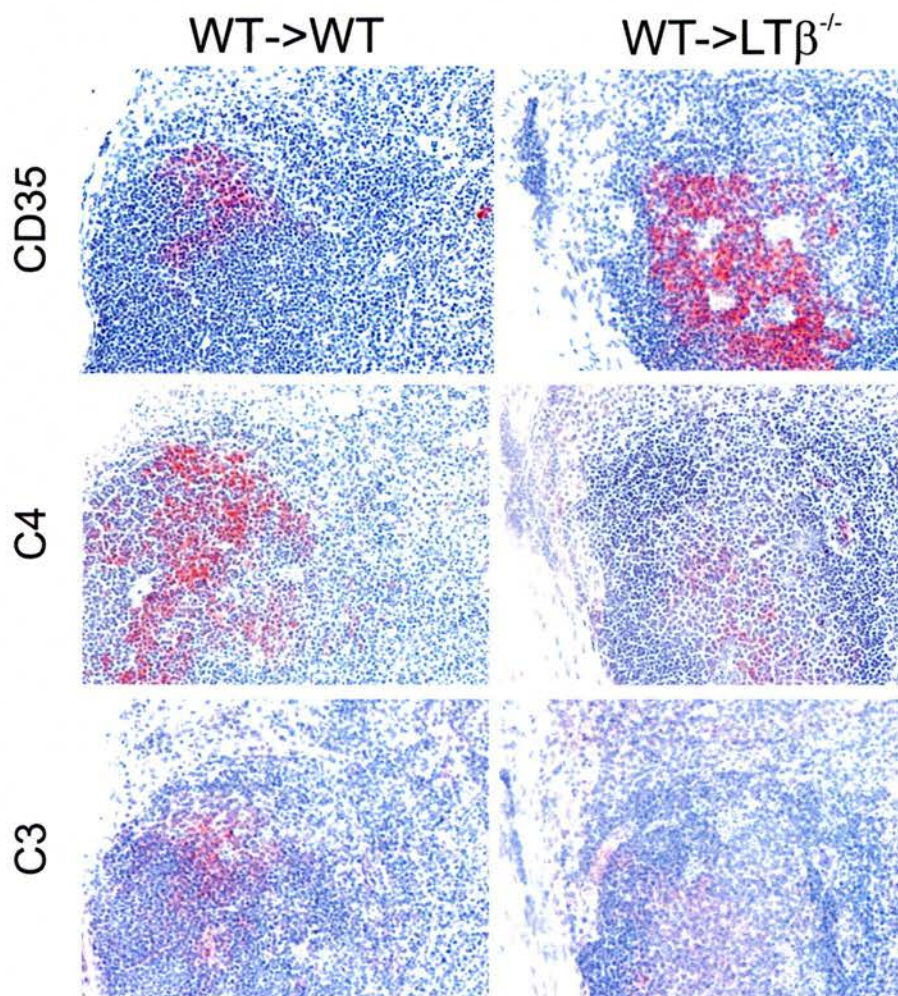


**Figure 4.7- Effect of reconstitution with wild-type (WT) bone marrow on the development mesenteric lymph nodes (MLNs) in recipient WT mice (WT->WT mice), lymphotoxin- $\alpha$  deficient mice (WT-> $LT\alpha^{-/-}$  mice) and lymphotoxin- $\beta$  deficient mice (WT-> $LT\beta^{-/-}$ ) mice. MLNs were present in WT mice, WT->WT mice,  $LT\beta^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice and absent from  $LT\alpha^{-/-}$  mice (data not shown) and WT-> $LT\alpha^{-/-}$  mice.**

the lymphoid tissues of WT->WT mice. Furthermore, the maturation of FDC networks and follicular organisation was induced in the remaining lymphoid tissues of WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice (Figs 4.1 & 4.2). The presence of FDCs at the time of oral inoculation has been found to be crucial for efficient scrapie agent accumulation in lymphoid tissues and subsequent neuroinvasion (Mabbott *et al.*, 2003). Here, the presence of immune complex-trapping FDCs at the time of inoculation in MLN and spleen tissues was shown by immunostaining for complement receptor (CR) 1 and complement components C3 and C4 (Figs 4.8 & 4.9). The expression of PrP<sup>C</sup> was similar in lymphoid tissues of WT->WT mice, WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice (Fig 4.10).

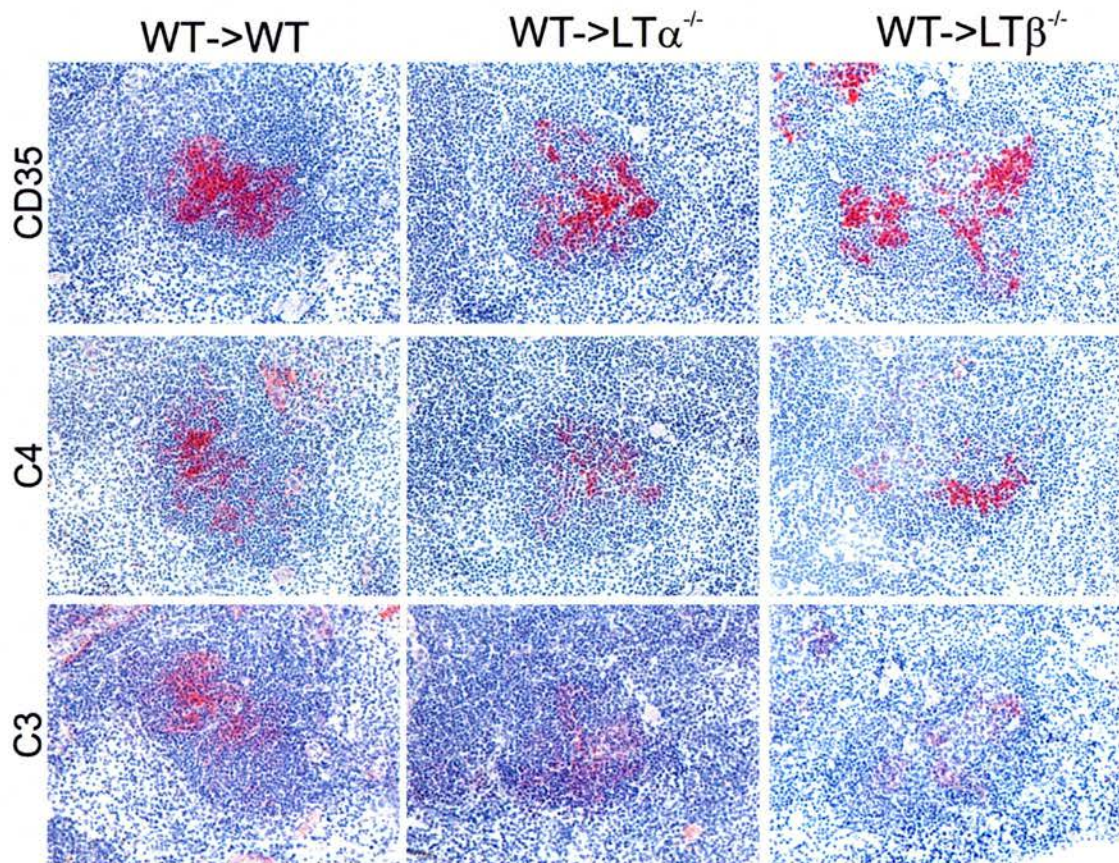
The reconstitution of LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice with WT bone marrow also induces the development of isolated lymphoid follicles (ILFs) in the intestine (Figs 3.5 & 3.6). Investigations in Chapter 3 of this thesis have revealed that ILF development in recipient LT $\alpha$ <sup>-/-</sup> mice or LT $\beta$ <sup>-/-</sup> mice takes much longer than 35 days, hence ILFs were absent in WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice 35 days after WT bone marrow transfer (Fig 3.6). In summary, at 35 days after bone marrow transfer WT->LT $\alpha$ <sup>-/-</sup> mice had no MLNs, PPs or ILFs and WT->LT $\beta$ <sup>-/-</sup> mice had no PPs or ILFs. However, in all three groups of mice (WT->WT mice, WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice) functional FDCs were present in the remaining lymphoid tissues (Fig 4.8 & 4.9).



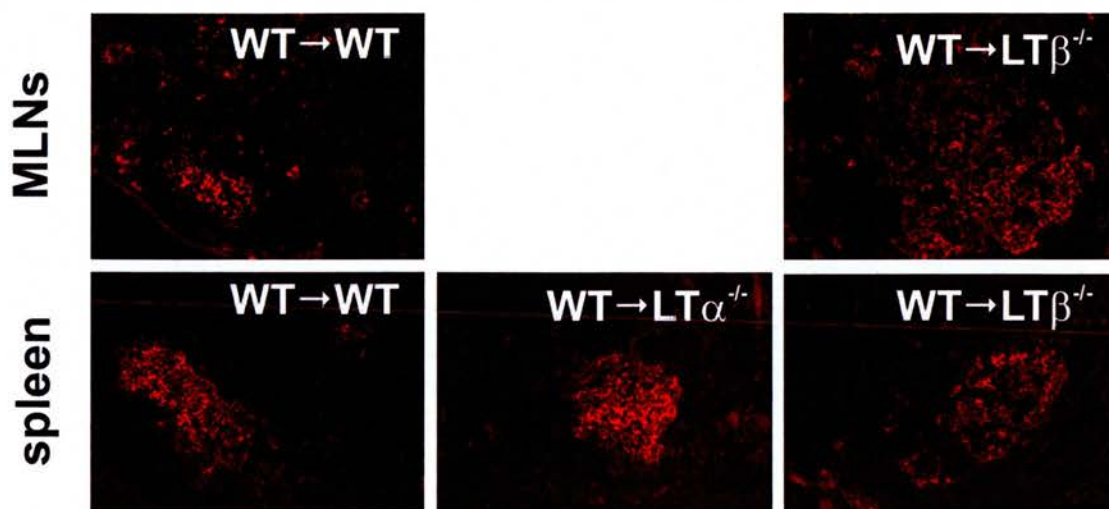


**Figure 4.8- Mesenteric lymph nodes (MLNs) from wild-type (WT) mice and lymphotoxin- $\beta$  deficient ( $LT\beta^{-/-}$ ) mice reconstituted with WT bone marrow (WT->WT mice and WT-> $LT\beta^{-/-}$  mice respectively) contain functional follicular dendritic cells (FDCs).** MLN tissues were collected at the time of oral inoculation with ME7 scrapie (5 weeks post reconstitution with WT bone marrow). Sections were stained with CD35-specific monoclonal antisera to detect FDCs (red; top row) and monoclonal antisera specific for complement components C4 (red; 2<sup>nd</sup> row) and C3 (red; lower row). Complement components in association with complement receptor expressing FDCs were found in the MLNs of both WT->WT mice (left) and WT-> $LT\beta^{-/-}$  mice (right).





**Figure 4.9-** Spleens from wild-type (WT) mice, lymphotoxin- $\alpha$  deficient mice ( $LT\alpha^{-/-}$ ) mice and lymphotoxin- $\beta$  deficient mice ( $LT\beta^{-/-}$ ) mice reconstituted with WT bone marrow (WT->WT mice, WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice respectively) contain functional follicular dendritic cells (FDCs). Spleen tissues were collected at the time of oral inoculation with ME7 scrapie (5 weeks post reconstitution). Sections were stained with CD35-specific monoclonal antisera to detect FDCs (red; top row) and monoclonal antisera specific for complement components C4 (red; 2<sup>nd</sup> row) and C3 (red; lower row). Complement components in association with complement receptor expressing FDCs were found in the spleens of WT->WT mice (left column), WT-> $LT\alpha^{-/-}$  mice (middle column) and WT-> $LT\beta^{-/-}$  mice (right column).



**Figure 4.10- Expression of the cellular form of the prion protein (PrP<sup>C</sup>) on follicular dendritic cells (FDCs) in lymphoid tissues of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively). Mesenteric lymph nodes (MLNs; where present) and spleen tissue sections were stained with the PrP-specific monoclonal antiserum 1B3 (red). Levels of PrP<sup>C</sup> expression were similar in all tissues examined. Original magnification X200.**



#### **4.3.5 Effect of GALT component-deficiency on oral scrapie susceptibility**

WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were  $\gamma$ -irradiated and reconstituted with WT bone marrow. 35 days later (when FDC networks had developed in remaining lymphoid tissues) mice were inoculated orally with the scrapie agent. Experiments demonstrated that all WT->WT mice succumbed to disease with a mean incubation period of  $337 \pm 4$  days ( $n = 8$ ). However, all WT-> $LT\alpha^{-/-}$  and WT-> $LT\beta^{-/-}$  mice remained free of the signs of scrapie up to at least 503 days after inoculation (Table 4.3). Characteristic spongiform pathology,  $PrP^d$  accumulation and reactive astrocytes were detected in the brains of all WT->WT mice that developed clinical signs of scrapie (Fig 4.11). The severity and distribution of the pathological targeting of vacuolation in the brains of WT mice was typical of ME7 scrapie (Fig 4.4). In contrast, no evidence of spongiform pathology,  $PrP^d$  accumulation or reactive astrocytes were detected in the brains of surviving WT-> $LT\alpha^{-/-}$  mice or WT-> $LT\beta^{-/-}$  mice (Fig 4.11).

#### **4.3.6 Effect of GALT deficiency on early and late $PrP^{Sc}$ accumulation in lymphoid tissues**

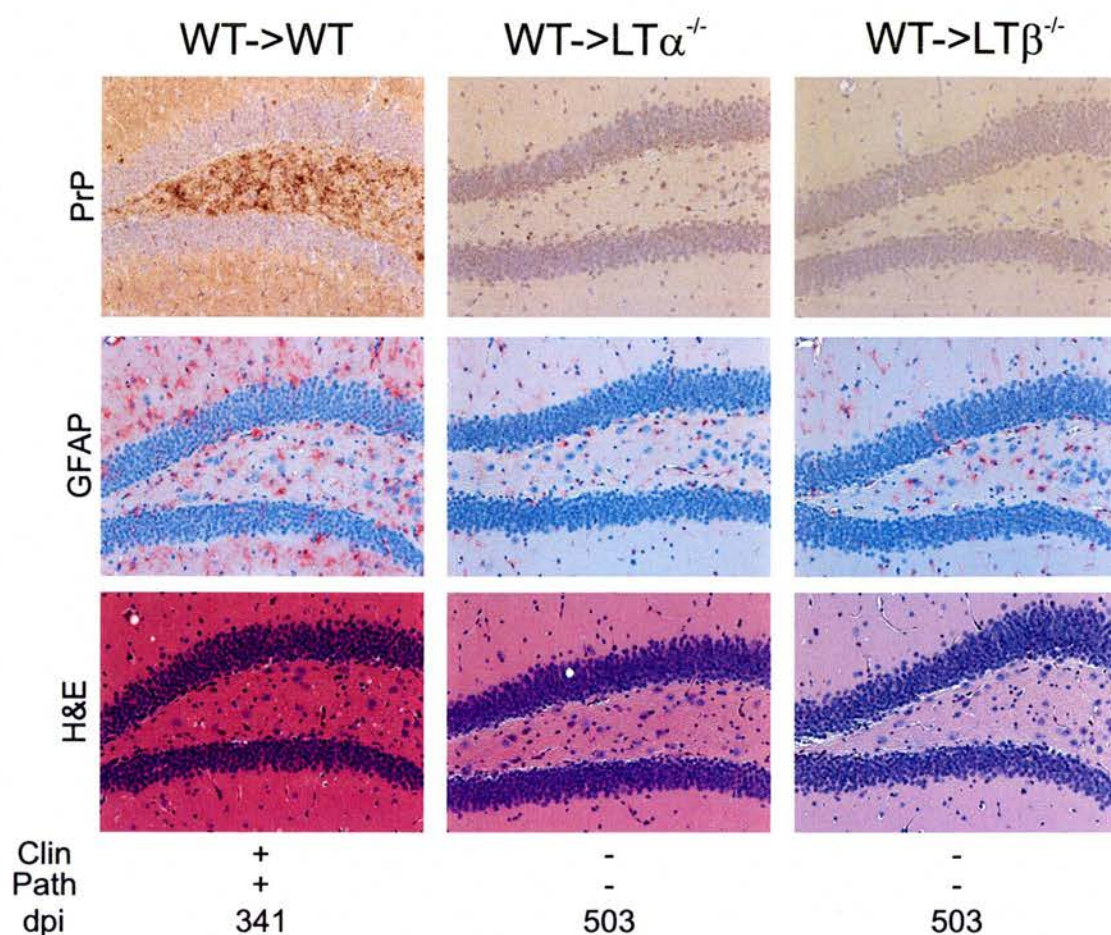
Within 70 days of oral inoculation with the ME7 scrapie agent high levels of  $PrP^d$  accumulate in association with FDCs in the germinal centres of PPs in the intestines (Mabbott *et al.*, 2003). High levels of  $PrP^d$  were found upon FDCs in the PPs and the few mILFs present in the intestines of WT->WT mice at 70 days after inoculation (Fig 4.12). After the early onset of accumulation, high levels of  $PrP^d$  were sustained in PPs and mILFs of WT->WT mice through to the terminal stages of disease (Fig



	Presence at time of inoculation				Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.
	SPL	MLNs	PPs	mILFs		
WT->WT	+	+	+	+	8/8	337 ± 4
WT->LTα <sup>-/-</sup>	+	-	-	-	0/8	5x > 503 <sup>a</sup>
WT->LTβ <sup>-/-</sup>	+	+	-	-	0/7	4x > 503 <sup>b</sup>

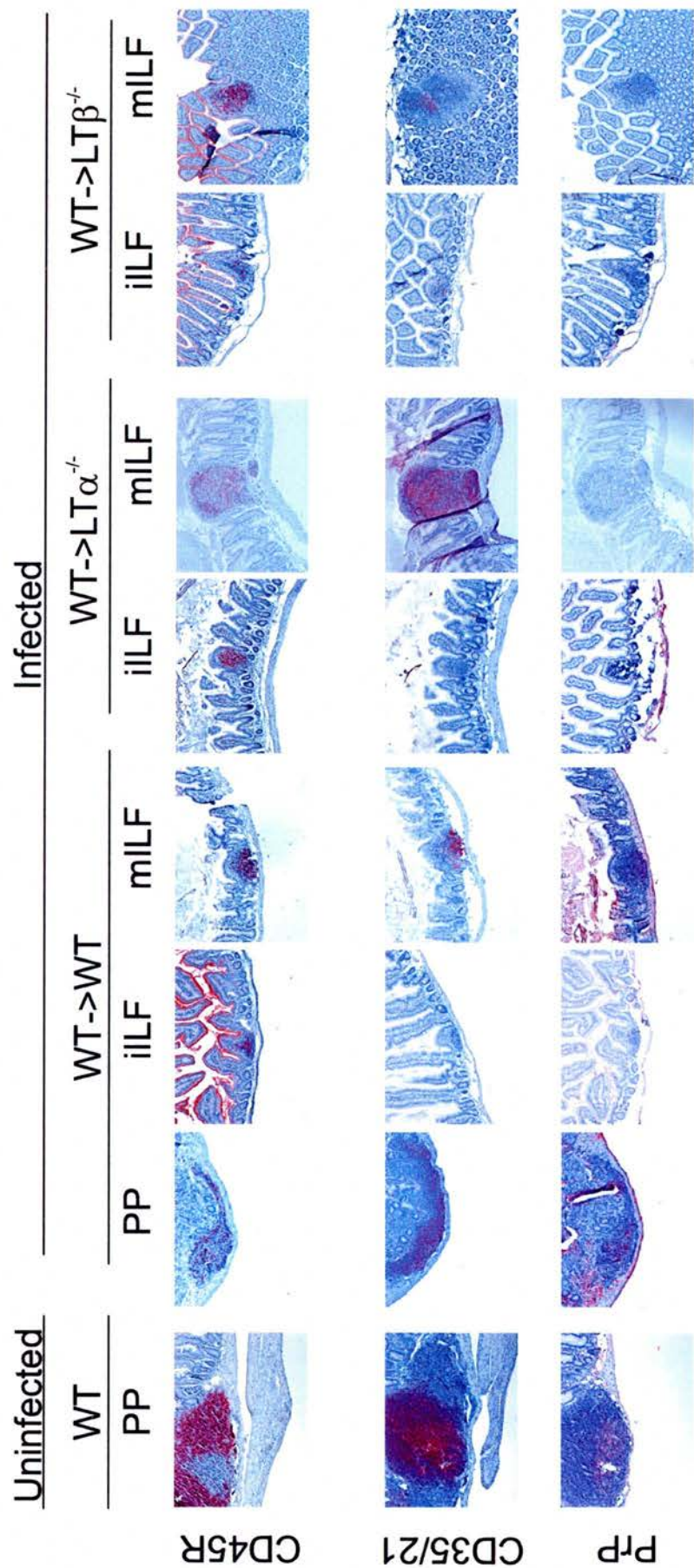
**Table 4.3 – Effect of Peyer’s patch (PP) and mature isolated lymphoid follicle (mILF) deficiency on scrapie susceptibility after oral inoculation.**

- Incidence = number of animals affected/ number of animals tested. The notation nx > 503 means that mice were free of the signs of scrapie up to at least this time point after inoculation
    - Three mice were killed 406, 450 and 467 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull and no histopathological signs of scrapie were detected in the brain (data not shown).
    - Three mice were killed 413, 455 and 455 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull and no histopathological signs of scrapie were detected in the brain (data not shown).
- SPL = Spleen, MLNs = mesenteric lymph nodes, PPs = Peyer’s patches, mILFs = mature isolated lymphoid follicles, FDCs = follicular dendritic cells, WT->WT, WT->LTα<sup>-/-</sup> and WT->LTβ<sup>-/-</sup> are wild-type, lymphotoxin -α deficient and lymphotoxin -β deficient mice respectively, reconstituted with WT bone marrow.
- + = presence, - = absence (for SPL, MLNs, PPs and FDCs)
- +/- = presence of few (<10) mILFs compared to + = presence of many (>10) mILFs when sections of intestine were examined microscopically.



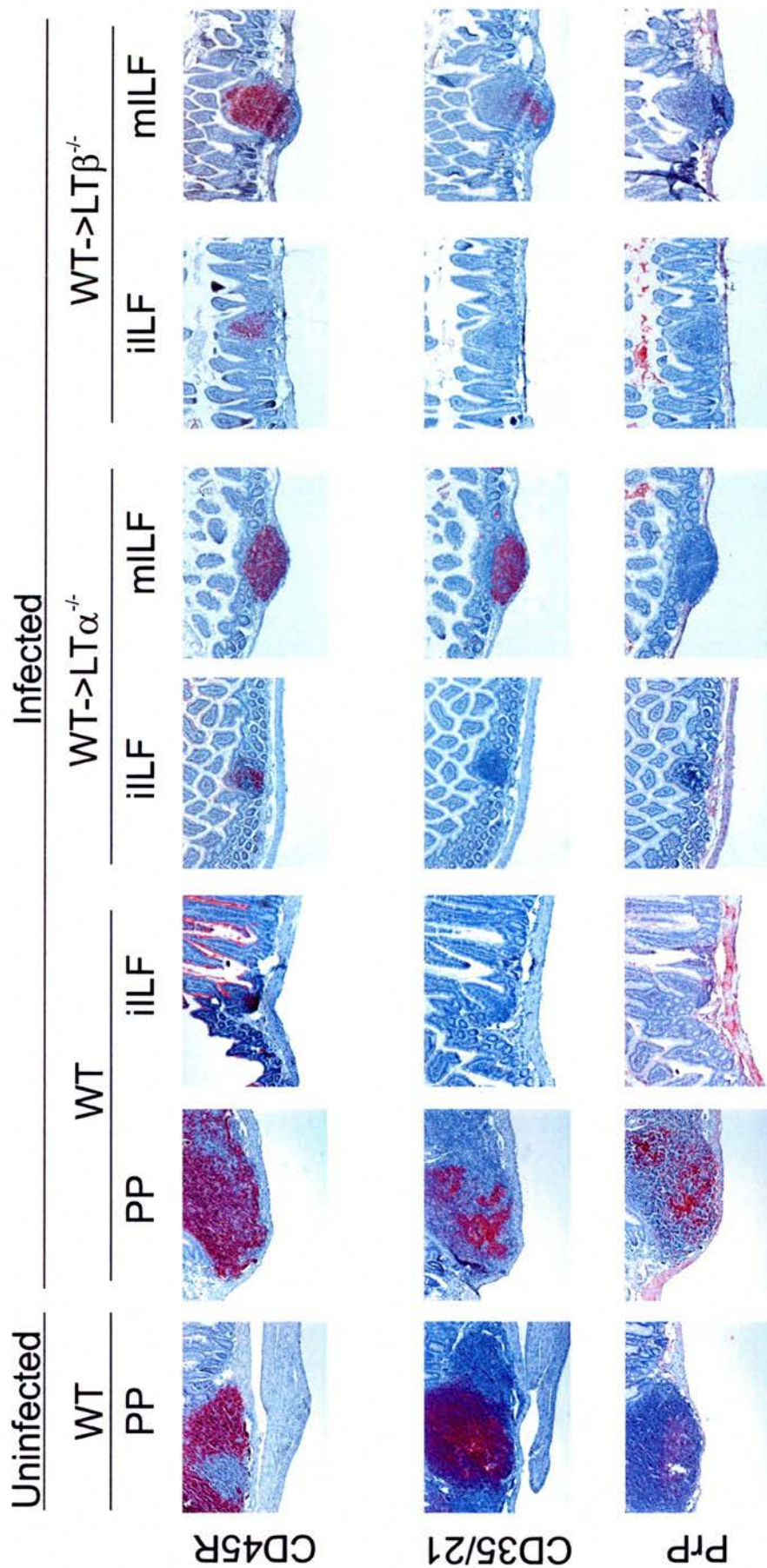
**Figure 4.11- Histological analysis of brain tissue from terminally scrapie affected mice or survivor mice.** Large disease-specific prion protein (PrP<sup>d</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-); Path, pathological score either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are wild-type mice, lymphotoxin- $\alpha$  deficient mice and lymphotoxin-  $\beta$  deficient mice respectively, reconstituted with wild-type bone marrow.



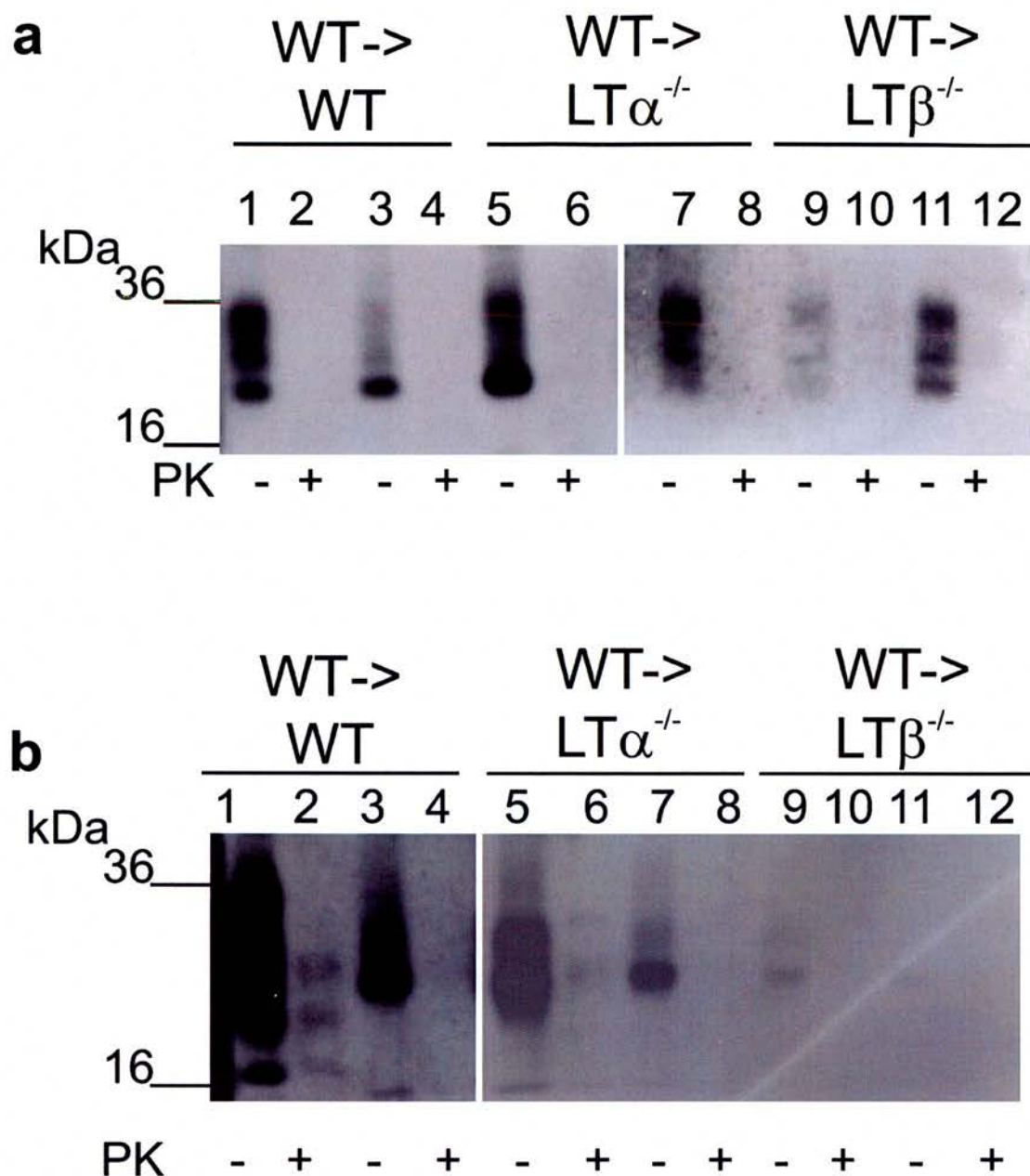


**Figure 4.12- Detection of disease specific prion protein (PrP<sup>d</sup>) in the intestines of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin- $\alpha$  deficient mice and lymphotoxin - $\beta$  deficient mice (WT->WT mice, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  mice respectively) 70 days after oral inoculation with ME7 scrapie. Serial sections were stained with anti-CD45R specific antiserum B220 to detect B lymphocytes (upper row; red), monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; middle row; red) and PrP specific antiserum 1B3 (bottom row; red). PrP staining was stronger in PPs from scrapie infected mice when compared to uninfected mice. Strong PrP<sup>d</sup> staining was found to co-localise with CD21/35 expressing FDCs in the B lymphocyte regions of Peyer's patches (PPs) and mature isolated lymphoid follicles (mILFs) of WT->WT mice. No PrP<sup>d</sup> staining was detected in mILFs of either WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice. Original magnification X100.**



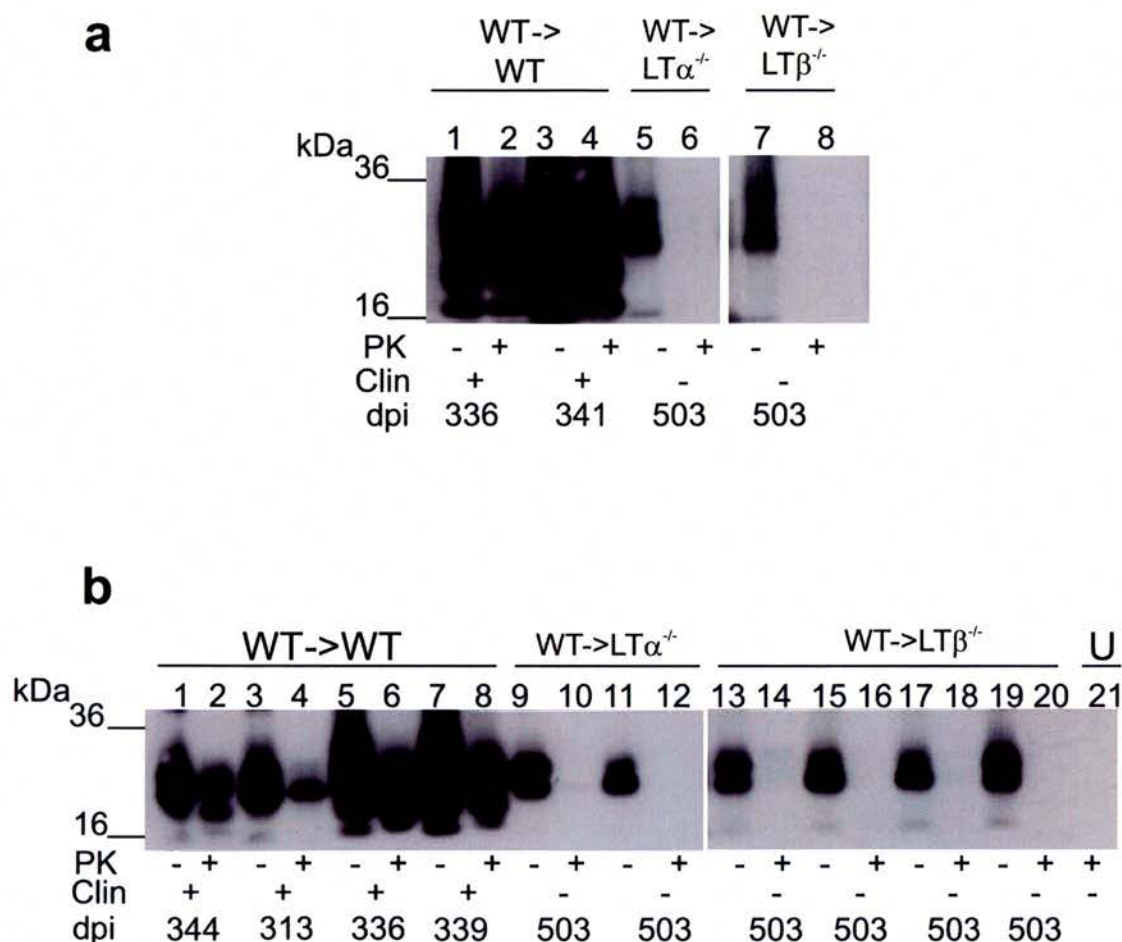


**Figure 4.13- Detection of disease specific prion protein (PrP<sup>d</sup>) in the intestines of clinically affected wild-type (WT) bone marrow reconstituted WT mice (WT->WT) mice and survivor WT bone marrow reconstituted lymphotoxin - $\alpha$  deficient mice and lymphotoxin - $\beta$  deficient mice (WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively) after oral inoculation with ME7 scrapie. Serial sections were stained with anti-CD45R specific antiserum B220 to detect B lymphocytes (upper row; red), monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; middle row; red) and PrP specific antiserum 1B3 (bottom row; red). PrP staining was stronger in Peyer's patches (PPs) from scrapie infected mice when compared to uninfected mice. Strong PrP<sup>d</sup> staining was found to co-localise with CD21/35 expressing FDCs in the B lymphocyte regions of PPs and mature isolated lymphoid follicles (mILFs) from WT->WT mice. No PrP<sup>d</sup> staining was detected in mILFs of survivor WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice taken 503 days after inoculation. Original magnification X100.**



**Figure 4.14- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) Mesenteric lymph nodes (MLNs; or membrane where MLNs should have been in WT->LT $\alpha^{-/-}$  mice) and (b) spleens of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin  $\alpha$ -deficient mice and lymphotoxin  $\beta$ -deficient mice (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively) 70 days after oral inoculation with ME7 scrapie. Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in some spleens from WT->WT mice. No PrP<sup>Sc</sup> was detected in MLNs from WT->WT mice or MLNs or spleens from either WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis.





**Figure 4.15- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) Mesenteric lymph nodes (MLNs; or membrane where MLNs should have been in WT->LT $\alpha^{-/-}$  mice) and (b) spleens of clinically affected wild-type (WT) bone marrow reconstituted WT mice (WT->WT mice), or survivor WT bone marrow reconstituted lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice (WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively) after oral inoculation with ME7 scrapie. Proteinase K resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in the MLNs and the spleens of WT->WT mice. No PrP<sup>Sc</sup> was detected in either MLNs or spleens from either WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice or from mice which were not inoculated with scrapie (U). Samples were treated in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis. Clin, clinical score either positive (+) or negative (-); dpi, days post inoculation on which the tissues were taken for analysis.



4.13). In contrast, no PrP<sup>d</sup> accumulations were observed in the mILFs in the intestines of either WT->LT $\alpha$ <sup>-/-</sup> mice or WT->LT $\beta$ <sup>-/-</sup> mice at 70 days after inoculation (Fig 4.12) or in the intestines of mice which remained free of the clinical signs of disease 503 days after inoculation (the end of the experiment; Fig 4.13). In the MLNs of WT->WT mice PrP<sup>Sc</sup> was undetectable 70 dpi (Fig 4.14) but strong accumulations were detected at the terminal stage of disease (Fig 4.15). Consistent with the observations made in ILFs, no PrP<sup>Sc</sup> was detected in the MLNs from WT->LT $\beta$ <sup>-/-</sup> mice 70 days after oral inoculation (Fig 4.14) or at the end of the experiment (Fig 4.15). In the spleens of WT->WT mice variable levels of PrP<sup>Sc</sup> were detected 70 dpi whereas high levels were detected at the terminal stage of disease (Fig 4.15). In contrast, PrP<sup>Sc</sup> was undetected in spleens from WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice 70 days after inoculation (Fig 4.14) or in spleens from clinically negative survivor WT->LT $\alpha$ <sup>-/-</sup> mice or WT->LT $\beta$ <sup>-/-</sup> mice (Fig 4.15).

#### **4.3.7 Effect of PP deficiency on oral scrapie pathogenesis in mice containing mILFs.**

The data described so far shows that the absence of PPs and mILFs at the time of inoculation blocks scrapie pathogenesis, whereas the presence or absence of MLNs does not influence scrapie susceptibility. Mice treated *in utero* with LT $\beta$ R-Ig on day 11.5 of gestation had no PPs, but retained the MLNs and had numerous mILFs in their intestines (Section 3.3.1). These mice (termed PP-deficient mice) were used here to determine the potential contribution of mILFs to oral scrapie pathogenesis. As the effects of LT $\beta$ R-Ig treatment are temporary, the remaining lymphoid tissues of PP-deficient mice (e.g. spleen and MLNs) had functional FDC networks at the

time of inoculation. When inoculated orally with the scrapie agent, immunocompetent control mice developed disease with a mean incubation period of  $319 \pm 2$  days ( $n = 16$ ). In contrast to the effects of PP and mILF deficiency on disease pathogenesis, scrapie susceptibility was not affected in PP-deficient mice. All PP-deficient mice developed disease with a mean incubation period of  $313 \pm 6$  days ( $n = 10$ ; Table 4.4). Histopathological analysis of brain tissue from clinically affected control mice and PP-deficient mice revealed the presence of characteristic PrP<sup>d</sup> accumulations, gliosis and spongiform pathology typical of mice terminally affected by the ME7 scrapie agent (Fig 4.16). No significant differences in the severity or distribution of the pathological targeting of vacuolation in the brain were observed between PP-deficient mice and control mice (Fig 4.17).

#### **4.3.8 Effect of PP deficiency on the early and late accumulation of PrP<sup>Sc</sup> in mice containing mILFs**

In the PPs and the few mILFs of control mice and in the mILFs of PP-deficient mice strong accumulations of PrP<sup>d</sup> were detected in association with FDCs 70 dpi (Fig 4.18) and were sustained through to the terminal stage of disease (Fig 4.19). Immunostaining with S-100 specific antiserum to detect neurones and supporting glial cells revealed the presence of neurones in close association with mILFs (Fig 4.20). 70 days after inoculation, PrP<sup>Sc</sup> accumulation was found some MLNs but not spleens of both PP-deficient mice and control mice (Fig 4.21). In terminally scrapie affected mice PrP<sup>Sc</sup> accumulations were found in both MLNs and spleens (Fig 4.22). Taken together, the results show that mILFs are capable of compensating for the PP

deficiency by supporting TSE agent accumulation in the intestine and facilitating subsequent neuroinvasion. Furthermore these data show that TSE agent neuroinvasion from mILFs is as efficient as neuroinvasion from other GALT.



	Presence at time of inoculation					Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.
	SPL	MLNs	PPs	mILFs	FDCs in remaining lymphoid tissues		
C57BL/Dk (control)	+	+	+	+/-	+	16/16	319 ± 2
PP-deficient C57BL/Dk	+	+	-	+	+	10/10	313 ± 6

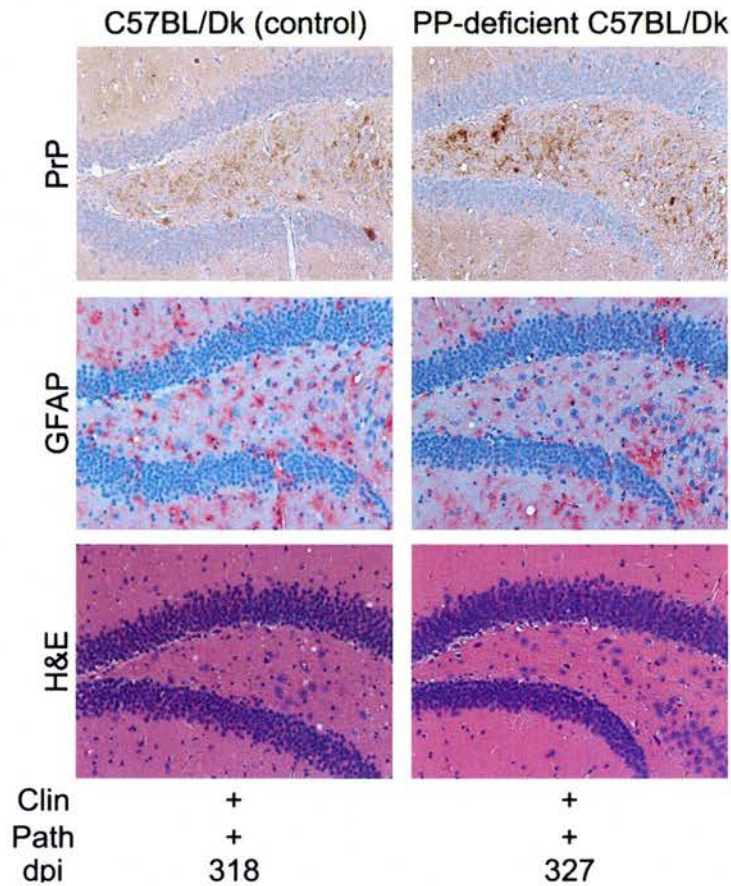
**Table 4.4 – Effect of PP deficiency on scrapie susceptibility after oral inoculation.**

1. Incidence = number of animals affected/ number of animals tested.

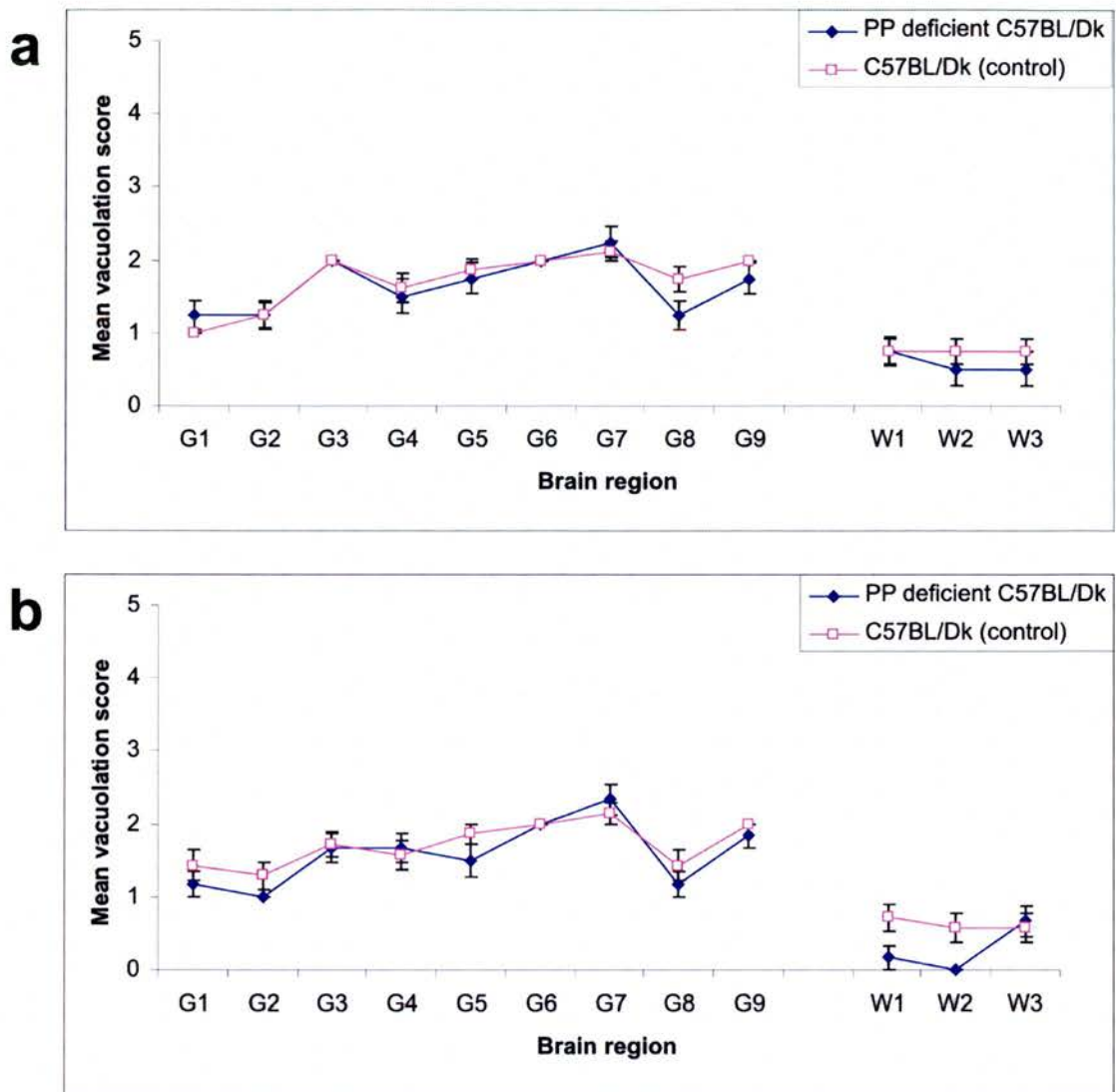
SPL = Spleen, MLNs = mesenteric lymph nodes, PPs = Peyer's patches, mILFs = mature isolated lymphoid follicles, FDCs = follicular dendritic cells

+ = presence, - = absence (for SPL, MLNs, PPs and FDCs)

+/- = presence of few (<10) mILFs compared to + = presence of many (>10) mILFs when sections of intestine were examined microscopically

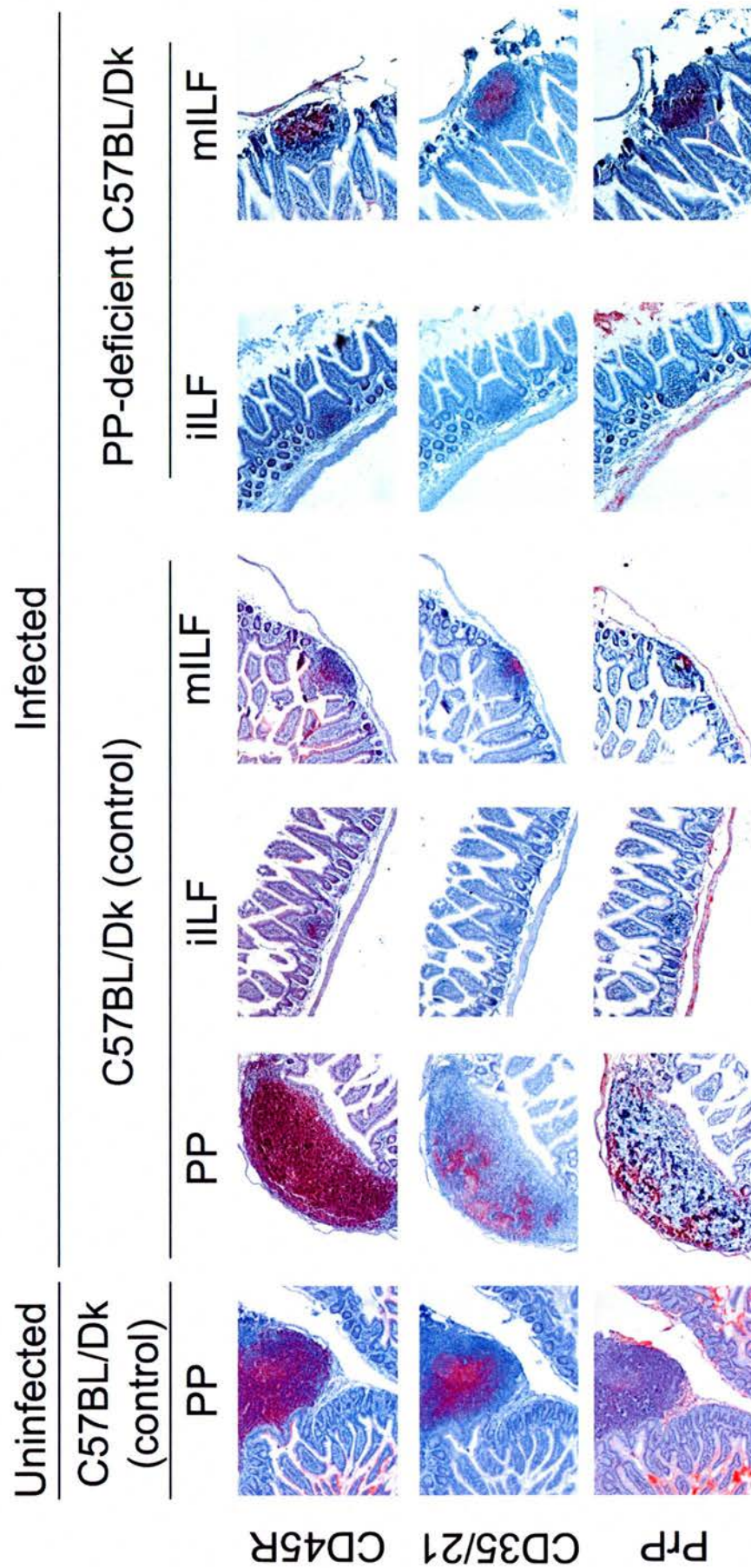


**Figure 4.16- Histological analysis of brain tissue from terminally scrapie affected mice.** Large disease specific prion protein (PrP<sup>d</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H&E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-); Path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis.



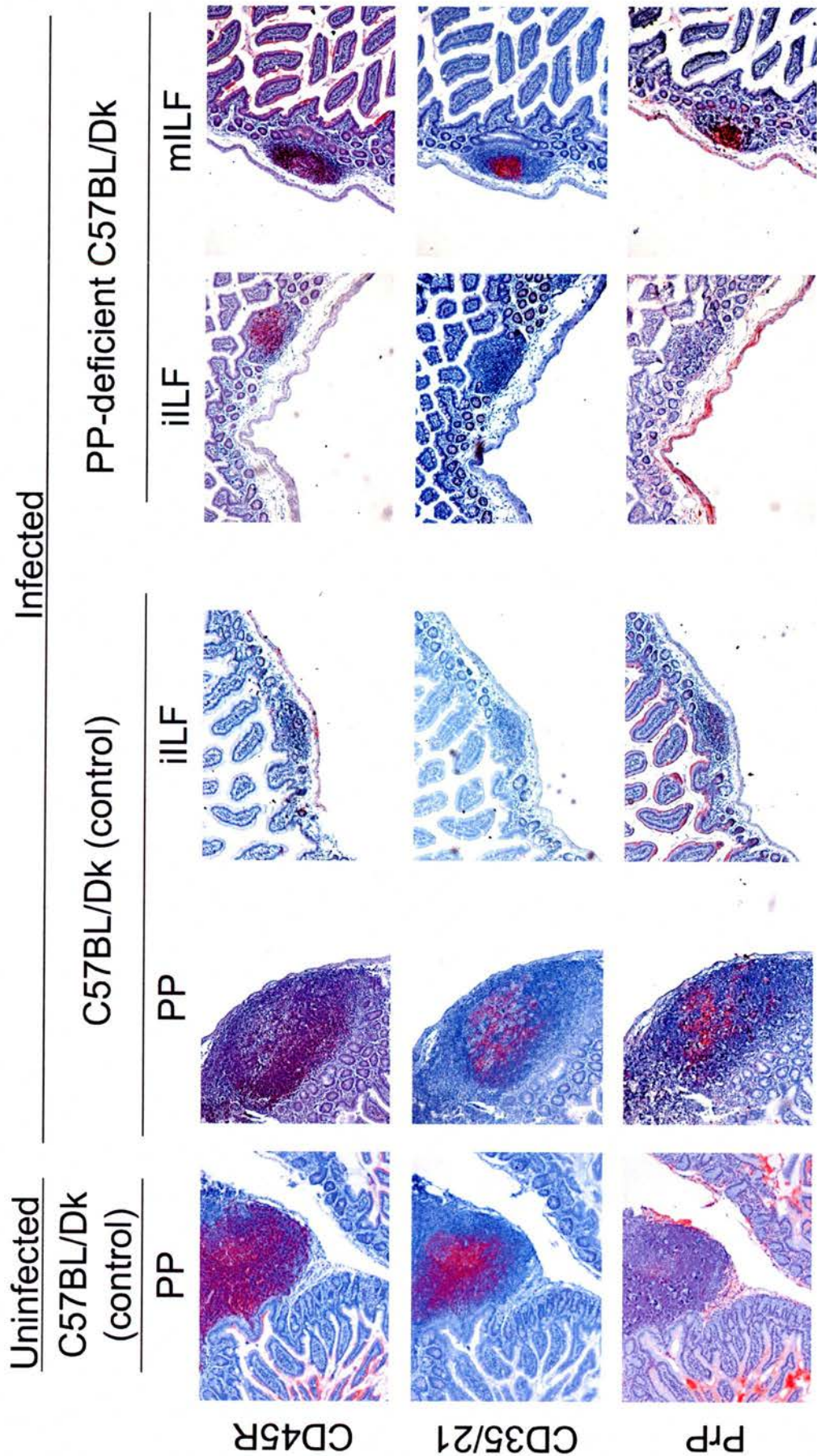
**Figure 4.17- Pathological targeting of vacuolation in the brains of mice terminally affected by ME7 scrapie. (a) first undertaking of experiment (b) second undertaking of experiment.** Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (G1-G9) or white matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of the superior peduncles; W3, cerebral peduncles. Each point represents mean vacuolation score  $\pm$  S.E.M. for groups of 6-9 mice



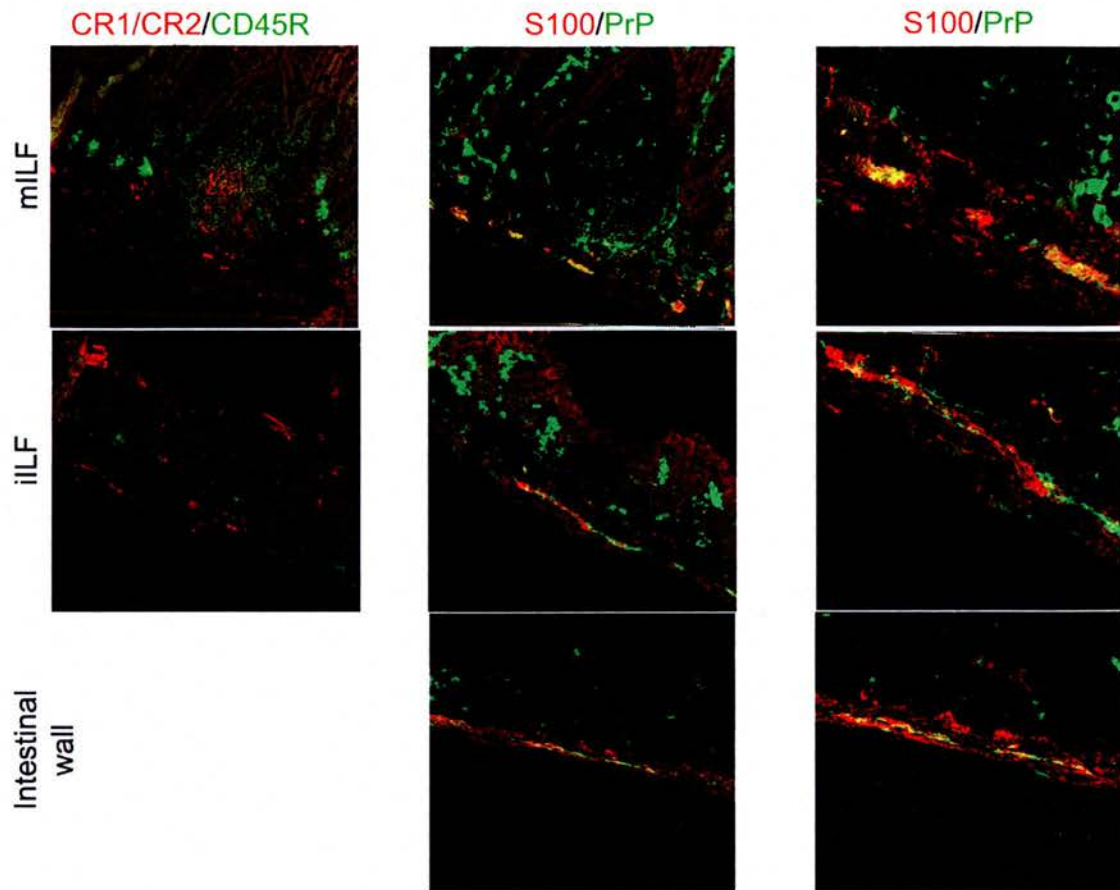


**Figure 4.18- Detection of disease specific prion protein (PrP<sup>sc</sup>) in the intestines of C57BL/Dk (control) mice and PP-deficient C57BL/Dk mice 70 days after oral inoculation with ME7 scrapie.** Serial sections were stained with anti-CD45R specific antiserum B220 to detect B lymphocytes (upper row; red), monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; middle row; red) and PrP specific antiserum 1B3 (bottom row; red). PrP staining was stronger in Peyer's patches (PPs) from scrapie infected mice when compared to uninfected mice. Strong PrP<sup>sc</sup> staining was found to co-localise with CD21/35 expressing FDCs in the B lymphocyte regions of PPs and mature isolated lymphoid follicles (mILFs). Original magnification X100.



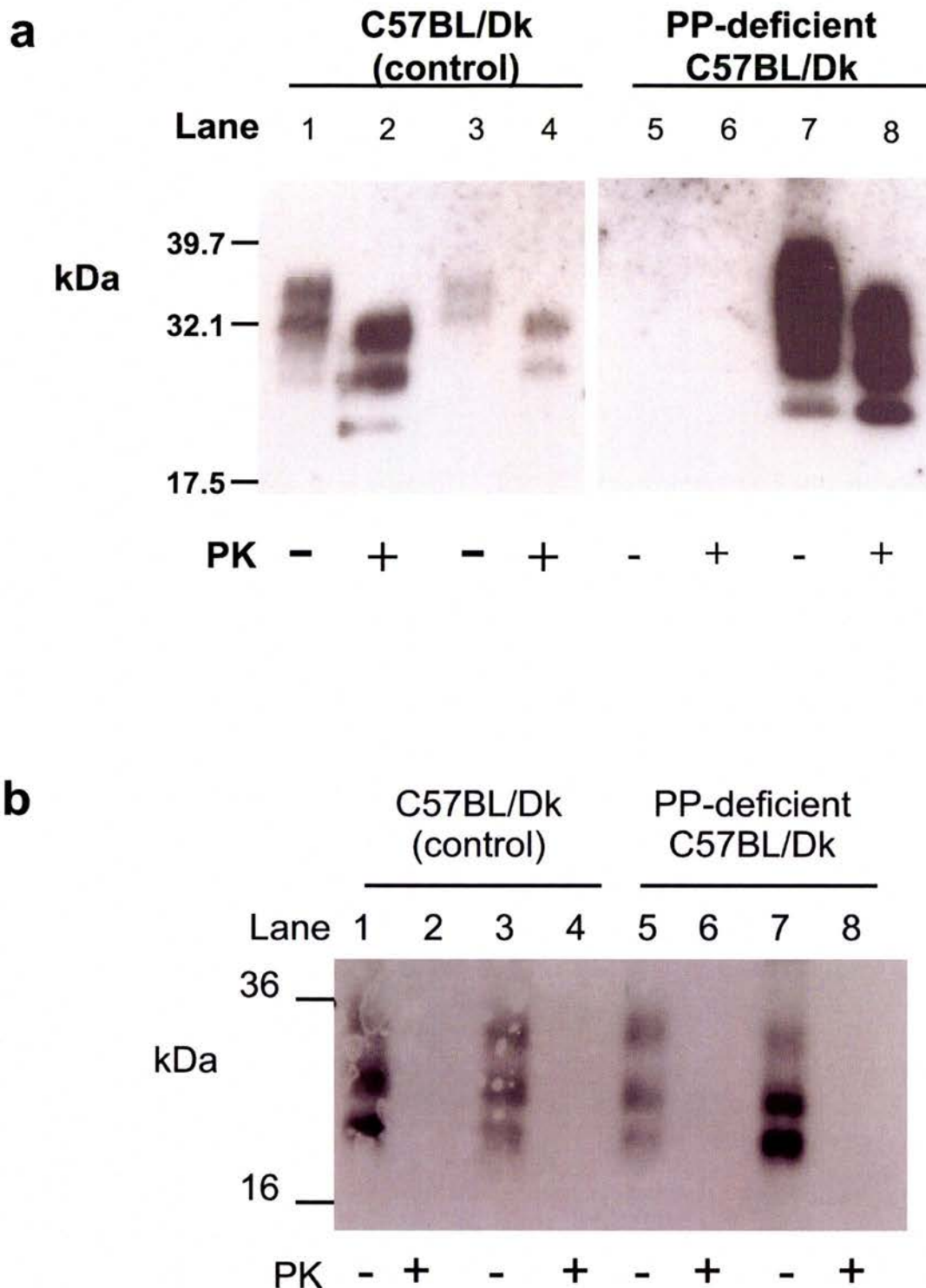


**Figure 4.19- Detection of disease specific prion protein (PrP<sup>d</sup>) in the intestines of clinically affected C57BL/Dk (control) mice and PP-deficient C57BL/Dk mice after oral inoculation with ME7 scrapie.** Serial sections were stained with anti-CD45R specific antiserum B220 to detect B lymphocytes (upper row; red), monoclonal antiserum 7G6 to detect CD21/35 expressing follicular dendritic cells (FDCs; middle row; red) and PrP specific antiserum 1B3 (bottom row; red). PrP staining was stronger in Peyer's patches (PPs) from infected mice when compared to uninfected mice. Strong PrP<sup>d</sup> staining was found to co-localise with CD21/35 expressing FDCs in the B lymphocyte regions of PPs and mature isolated lymphoid follicles (mILFs). Original magnification X100.

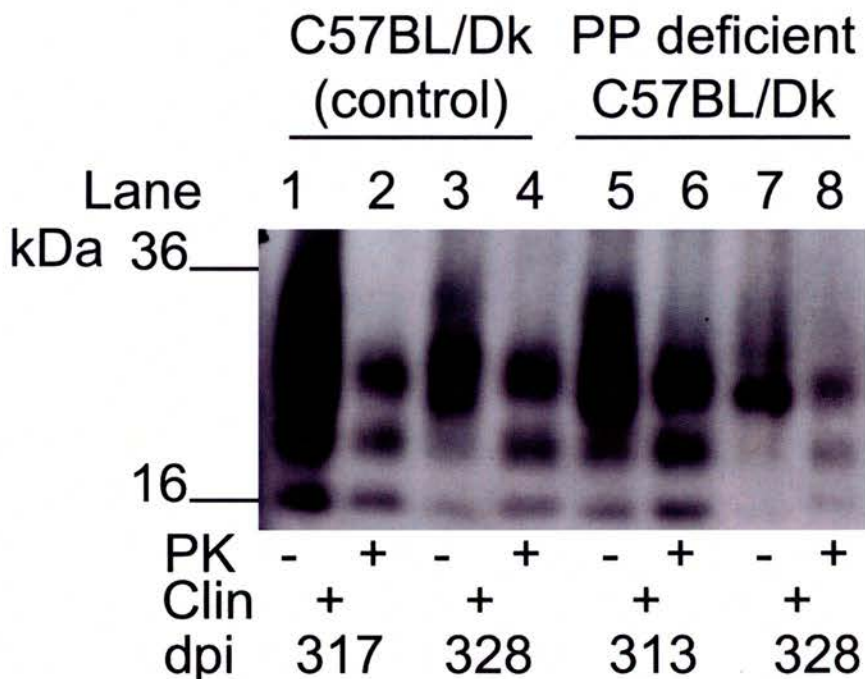
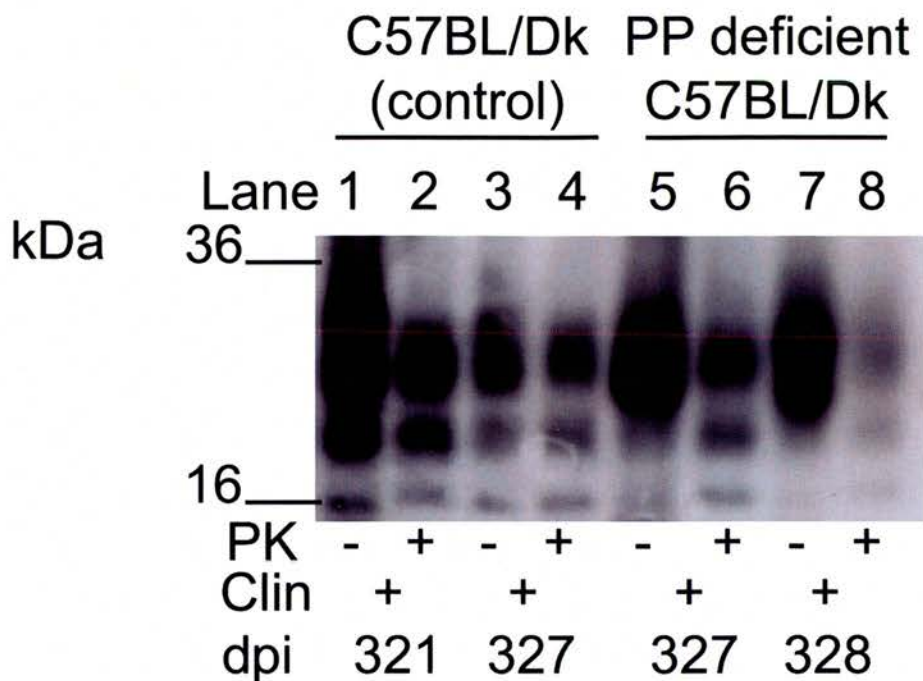


**Figure 4.20- Association of neurones with isolated lymphoid follicles (ILFs).** Sections of small intestine were stained with the CD45R-specific monoclonal antiserum B220 (green) and 7G6 monoclonal antiserum to detect CD21/35 expressing follicular dendritic cells (FDCs; red; left column). Adjacent sections were stained with the prion protein (PrP) -specific monoclonal antisera 6H4 (green) and S-100 specific monoclonal antisera to detect nerve fibres and supportive glial cells (red; middle and right columns). Neurones were found in close association with both mature ILFs (mILFs; top row) and immature ILFs (iILFs; lower row). Original magnification X200 (left and middle columns) and X630 (right column)





**Figure 4.21- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) Mesenteric lymph nodes (MLNs) and (b) spleens of C57BL/Dk (control) mice and PP-deficient C57BL/Dk mice 70 days after oral inoculation with ME7 scrapie. Samples were treated in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis. At this time point, PK resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in some MLNs but not in the spleens.



**Figure 4.22- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) mesenteric lymph nodes (MLNs) and (b) spleens of clinically affected C57BL/Dk (control) mice and Peyer's patch (PP)-deficient C57BL/Dk mice after oral inoculation with ME7 scrapie. Proteinase K resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in both the MLNs and the spleens. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score, either positive (+) or negative (-); dpi; days post inoculation on which the tissues were taken for analysis.

#### **4.4 Discussion**

Mice lacking FDCs and specific combinations of GALT were inoculated orally with the ME7 scrapie agent to investigate the contribution of individual lymphoid tissues in scrapie pathogenesis after oral inoculation.  $LT\alpha^{-/-}$  mice lack PPs and MLNs,  $LT\beta^{-/-}$  mice lack PPs and neither have FDCs in the remaining lymphoid tissues. Consistent with the important role for FDCs in TSE agent neuroinvasion, none of the  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice succumbed to clinical disease following oral inoculation. Reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow induces the maturation of FDCs and microarchitecture in the remaining lymphoid tissues (for example the spleen), but does not induce the development of the missing PPs or MLNs. After reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow none of the recipient mice succumbed to disease. Thus in the absence of PPs and mILFs disease susceptibility was reduced. These results also suggest MLNs are not important for oral scrapie pathogenesis even though early TSE agent accumulation does occur in these tissues. Progeny mice treated *in utero* with  $LT\beta$ R-Ig lack PPs but contain increased numbers mILFs compared to untreated control mice. Furthermore, the total area covered by FDCs in the intestines of PP-deficient mice was equivalent to the total area covered by FDCs in immunocompetent control mice (Fig 3.5). Both PP-deficient mice and control mice succumbed to clinical scrapie at the same time and neuroinvasion occurred via the same pathway. The increased numbers of mILFs found in PP-deficient mice compensated for the PP deficiency by supporting neuroinvasion. Taken together these results show that lymphoid tissue in the intestine itself is important for effective pathogenesis of scrapie following oral inoculation.



In an adult mouse,  $LT\alpha_1\beta_2$  signalling through  $LT\beta$  receptor ( $LT\beta R$ ) is required for the terminal differentiation of FDCs and for the organisation of the microarchitecture of lymphoid tissues. Thus the remaining lymphoid tissues in mice genetically deficient in either  $LT\alpha$  or  $LT\beta$  also lack FDCs and show disorganised microarchitecture (Koni *et al.*, 1997; Matsumoto *et al.*, 1996).  $\gamma$ -irradiation and WT bone marrow reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice results in the restoration of FDC networks and organisation of lymphoid tissue microarchitecture demonstrating the necessity of LT expressing bone marrow derived cells for both these processes.

As discussed in section 3.4,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack PPs and ILFs as  $LT\alpha_1\beta_2$  signalling is required for the formation of these tissues. The mechanism of formation of MLNs is distinct from the  $LT\alpha_1\beta_2$  dependent mechanism involved in the formation of most other secondary lymphoid tissues as demonstrated by the presence of MLNs in  $LT\beta^{-/-}$  mice (Koni *et al.*, 1997) and *in utero*  $LT\beta R$ -Ig treated mice (Rennert *et al.*, 1997). The absence of MLNs from  $LT\alpha^{-/-}$  mice (Banks *et al.*, 1995; Fu *et al.*, 1997) and  $LT\beta R^{-/-}$  mice (Futterer *et al.*, 1998) and comparison of the levels of  $LT\beta R$  expressed by the cells present in MLN anlage compared to other lymph node anlage (Cupedo *et al.*, 2004b) gives some indication that in the absence of  $LT\alpha_1\beta_2$ , MLNs may form by the interaction of  $LT\alpha_3$  with  $LT\beta R$ . However, there is no published evidence to suggest that  $LT\alpha_3$  is a ligand for  $LT\beta R$ .  $\gamma$ -irradiation and WT bone marrow reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice does not restore the formation of the missing PPs and MLNs as the cells required to provide the source of LT for this process are no longer able to carry out this function in adult mice (Cupedo *et al.*,

2004a). FDCs are present and lymphoid tissue microarchitecture is normal in the remaining lymphoid tissues of mice treated *in utero* with LT $\beta$ R-Ig (Rennert *et al.*, 1996). In this instance LT signalling was temporarily blockaded during gestation and was restored following catabolism of LT $\beta$ R-Ig promoting subsequent FDC maturation in the progeny mice.

LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice have previously been used to investigate the importance of FDCs in the oral pathogenesis of TSEs (Oldstone *et al.*, 2002). This study found LT $\alpha$ <sup>-/-</sup> to be less susceptible than controls, and LT $\beta$ <sup>-/-</sup> mice to be resistant to oral RML scrapie agent challenge suggesting an important role for FDCs in oral scrapie pathogenesis. Studies have also investigated the importance of FDCs in TSE pathogenesis in other peripheral routes of inoculation using LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice (Manuelidis *et al.*, 2000; Prinz *et al.*, 2002). The results of these studies do not concur as the first found no difference in susceptibility between LT $\beta$ <sup>-/-</sup> mice and WT mice when inoculated intra-peritoneally with a mouse passaged strain of CJD and the second found both LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice to have decreased susceptibility and increased incubation periods to intra-peritoneal inoculation with RML scrapie. These differences could be explained by the different strains of TSE agent and different doses of inocula used. As these results are not consistent, and neither used the ME7 strain of TSE agent, the susceptibility of LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice to oral ME7 scrapie was investigated in this study. Both LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice were found to be resistant to oral ME7 scrapie which demonstrates that the combined absence of both FDCs and draining lymphoid tissue prevents the onset of ME7 scrapie after oral inoculation.

Other studies have also shown that in mouse models which had a combined lack of both PPs and FDCs ( $\text{TNF}\alpha^{-/-}$  x  $\text{LT}\alpha^{-/-}$  mice and *aly/aly* mice; Horiuchi *et al.*, 2006; Prinz *et al.*, 2003b), or atrophied PPs with reduced FDC networks ( $\beta 7^{-/-}$  mice,  $\text{RAG-1}^{-/-}$  mice and  $\mu\text{MT}^{-/-}$  mice; Prinz *et al.*, 2003b) scrapie disease did not develop after oral inoculation. However, in each study, the separate roles that lymphoid tissues and FDCs play in scrapie pathogenesis could not be distinguished. In this chapter, by reconstituting  $\text{LT}\alpha^{-/-}$  mice and  $\text{LT}\beta^{-/-}$  mice with WT bone marrow and thus restoring terminal FDC differentiation, data presented here shows the effect a specific lack of lymphoid tissue has on pathogenesis of ME7 scrapie after oral inoculation.

Both  $\text{WT} \rightarrow \text{LT}\alpha^{-/-}$  mice and  $\text{WT} \rightarrow \text{LT}\beta^{-/-}$  mice lacked PPs and mILFs at the time of oral inoculation (35 days after WT bone marrow reconstitution).  $\text{WT} \rightarrow \text{LT}\alpha^{-/-}$  mice and  $\text{WT} \rightarrow \text{LT}\beta^{-/-}$  mice were resistant to oral scrapie challenge, demonstrating the crucial importance of intestinal lymphoid tissue in oral scrapie pathogenesis. This result is unlikely to be due to impaired uptake of the scrapie agent as  $\text{WT} \rightarrow \text{LT}\alpha^{-/-}$  mice and  $\text{WT} \rightarrow \text{LT}\beta^{-/-}$  mice were found to have M cells and an intact lymphatic vasculature (Figs 3.12 & 3.13). Macrophages appear to be unaffected by lymphotoxin deficiency and although  $\text{LT}\alpha_1\beta_2$  signalling via  $\text{LT}\beta\text{R}$  plays an important role in the migration of DCs into lymphoid tissues, this defect is rectified by reconstitution with WT bone marrow (Kabashima *et al.*, 2005; Wu *et al.*, 1999).

Previous work has shown the presence of FDCs at the time of oral inoculation with the scrapie agent to be crucial for the establishment of disease, as mice lacking FDCs at the time of inoculation were resistant to disease despite the re-appearance of FDCs



around 28 days later (Mabbott *et al.*, 2003). Consistent with this, data presented in this chapter show that mice lacking PPs and mILFs at the time of inoculation did not succumb to disease despite the induction of FDC-containing mILFs around 28 days later (chapter 3). Data presented here also show, further to previous work, that FDCs must be present in the intestine at the time of inoculation as FDCs were present in other lymphoid tissues (e.g. MLNs and spleen) but these tissues were unable to support neuroinvasion. These data are consistent with data from an epidemiological, mathematical and pathological study which suggested that for sheep, cattle and humans there was an association between the development of lymphoid tissues in the gastrointestinal tract and susceptibility to natural TSE infection (St. Rose *et al.*, 2006).

The fact that WT->LT $\beta^{-/-}$  mice had MLNs containing functional FDCs at the time of inoculation but the mice did not develop disease demonstrates that although early PrP<sup>Sc</sup> accumulation can occur in MLNs, these tissues are not critical for oral scrapie pathogenesis. Additionally, although high levels of PrP<sup>d</sup> were detected in PPs 70 days after inoculation of WT->WT mice with scrapie, no PrP<sup>Sc</sup> was found in the MLNs at this time point. Taken together, these data indicate that MLNs are not critical sites of scrapie neuroinvasion.

The demonstration that neuroinvasion does not occur in mice which lack lymphoid tissue in the intestine, despite the presence of FDC-containing MLNs and spleen, implies that after oral inoculation, neuroinvasion most likely occurs directly from within the intestines. However, these experiments do not exclude the possibility that

the lack of an initial site for accumulation and replication of the scrapie agent merely prevents the onward spread of the agent and that the crucial neuroinvasion could be taking place from sites outwith the intestines, for example, the spleen. The early detection of PrP<sup>Sc</sup> in the enteric nervous system of hamsters inoculated orally with scrapie (Beekes & McBride, 2000) and the fact that splenectomy had no effect on pathogenesis of 139A scrapie after intragastric inoculation of mice (Kimberlin & Walker, 1989) gives some indication that neuroinvasion does occur directly from the intestines after oral inoculation. The presence of FDCs at the time of oral scrapie inoculation is critical for efficient neuroinvasion (Table 4.2; Mabbott *et al.*, 2003). However, treatment of mice with LT $\beta$ R-Ig to temporarily de-differentiate FDCs 14 days after oral inoculation with the ME7 scrapie agent had no effect on pathogenesis (Mabbott *et al.*, 2003). Together these data imply that crucial neuroinvasion occurs at a very early stage post inoculation and most likely from lymphoid tissue directly within the intestine.

TSE infectivity has been found in the absence of the detection of PrP<sup>Sc</sup> in mice infected with BSE and transgenic mice infected with scrapie (Barron *et al.*, 2001; Lasmezas *et al.*, 1997). The experiments described here do not exclude the possibility that there may have been infectivity present, albeit at low levels, in the absence of PrP<sup>Sc</sup> in the lymphoid tissues or brains of the mice which did not develop clinical disease. In experiments described in this thesis, PrP<sup>Sc</sup> was extracted from lymphoid tissues using a method which involved precipitation with sodium phosphotungstic acid (NaPTA) allowing the highly sensitive detection of low levels of PrP<sup>Sc</sup> (Safar *et al.*, 1998; Wadsworth *et al.*, 2001). This would suggest that if

infectivity was present in samples where no PrP<sup>Sc</sup> was detected the level is likely to be extremely low. ME7 is a very well characterised experimental strain of scrapie and the presence of PrP<sup>Sc</sup> is a reliable biochemical marker for infectivity for this strain of TSE agent (Mabbott *et al.*, 2003; Mohan *et al.*, 2005a). Thus, it is highly likely in the experiments described here that the absence of PrP<sup>Sc</sup> and clinical signs of disease equates to a lack of infectivity.

As discussed in section 3.4, the development of both PPs and ILFs is critically dependent upon LT $\alpha_1\beta_2$  signalling through LT $\beta$ R during gestation. However, differences in the timing and the type of cells that provide the source of LT permits ILFs to develop in mice treated *in utero* with LT $\beta$ R-Ig, but prevents the development of PPs (section 3.4). PP-deficient mice had increased numbers of FDC-containing mILFs compared with untreated control mice such that the total area covered by FDCs in the intestines of these two types of mice were equivalent (section 3.3.3). After oral inoculation with the scrapie agent both PP-deficient mice and control mice developed clinical disease with similar incubation periods. Furthermore, PrP<sup>Sc</sup> accumulation in remaining MLNs and spleens, and brain pathology were also similar in clinically affected PP-deficient and control mice. This suggests that neuroinvasion occurred via a common pathway and was not influenced if the initial accumulation of the scrapie agent occurred in PPs or mILFs. PrP<sup>d</sup> accumulated in association with FDCs in mILFs and neurones and supportive glial cells were found in close association with mILFs in the intestines of PP-deficient mice. Thus it is tempting to speculate that following accumulation upon FDCs within mILFs scrapie agent neuroinvasion occurred rapidly via the local enteric nerves.



Data presented here showed that scrapie agent accumulation upon FDCs in lymphoid tissue within the intestines (such as PPs or mILFs) was of crucial importance for the successful development of clinical disease after oral inoculation. Although early PrP<sup>Sc</sup> accumulation also occurs within the MLNs their absence did not affect disease susceptibility. These data demonstrate the crucial importance of the initial accumulation and replication phase in lymphoid tissue local to the site of inoculation for the development of clinical disease.

## Role of draining lymphoid nodes in scrapie pathogenesis after inoculation via scarification of the skin

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## **5.1 Abstract**

Inoculation with the scrapie agent via scarification of the skin has been shown to be an effective route for the establishment of scrapie disease. Mice lacking inguinal lymph nodes (ILNs) were inoculated with the scrapie agent via scarification of the skin of the thigh to investigate the role of the draining lymph nodes in scrapie pathogenesis. Lymphotoxin (LT)  $\alpha$  deficient mice ( $LT\alpha^{-/-}$ ) mice and  $LT\beta^{-/-}$  mice were reconstituted with wild-type bone marrow to create mice which did not possess ILNs but possessed follicular dendritic cells (FDCs) in remaining lymphoid tissues. ILN-deficient mice were also created by *in utero* treatment with  $LT\beta$ R-Ig which prevents the formation of the ILN in progeny mice. All groups of ILN-deficient mice displayed decreased susceptibility to scrapie after inoculation with the ME7 scrapie agent via scarification. These data demonstrate that the draining lymph nodes are important for efficient TSE neuroinvasion after inoculation via the skin.



## **5.2 Introduction**

Although many natural TSE infections are thought to be acquired orally, other peripheral routes of exposure have been identified. Many examples of iatrogenic transmission have been documented including; the transplantation of CJD contaminated tissues or hormones (Duffy *et al.*, 1974), or the transfusion of vCJD contaminated blood (Llewelyn *et al.*, 2004; Peden *et al.*, 2004). Surgical instruments contaminated with sCJD also have the potential to transmit disease (Flechsigs *et al.*, 2001). Experimental inoculation of mice has revealed scarification of the skin to be an effective means of scrapie transmission (Taylor *et al.*, 1996b). Scarification of the skin involves the creation of a superficial abrasion in the top layers of the skin into which the scrapie agent inoculum is administered (section 2.4.4). The effectiveness of this as a route for the transmission of scrapie raises the possibility that some cases of natural scrapie may arise through skin lesions during close contact between infected and non-infected animals, or through lesions in the mouth or digestive tract (Bartz *et al.*, 2003; Brotherston *et al.*, 1968). This also raises the possibility that pharmaceutical or cosmetic products derived from sheep or cattle tissues may harbour TSE infectivity and therefore pose a risk when applied to abraded skin (Birmingham, 2000; Lupi, 2002).

The involvement of lymphoid tissues in TSE pathogenesis after inoculation via scarification has been demonstrated by the resistance of severe combined immunodeficient (SCID) mice to scrapie administered in this way (Taylor *et al.*, 1996b). After inoculation via this route, neuroinvasion does not occur directly from nerves within the skin but occurs following accumulation in lymphoid tissues

(Mohan *et al.*, 2004; Mohan *et al.*, 2005a). The transport of the scrapie agent to the lymphoid tissues from the skin appears to be independent of Langerhans cells (Mohan *et al.*, 2005b), but this does not exclude a role for other subsets of migratory dendritic cells within the skin such as dermal dendritic cells (Lenz *et al.*, 1993). Further analysis has shown that follicular dendritic cells (FDCs) within the lymphoid tissue are important in this route of infection as the de-differentiation of FDCs shortly before or just after inoculation with scrapie extended survival time and reduced the incidence of disease (Mohan *et al.*, 2005a). The expression of PrP<sup>C</sup> by lymphocytes is unimportant for pathogenesis via this route as SCID mice grafted with PrP<sup>+/+</sup> bone marrow and SCID mice reconstituted with PrP<sup>-/-</sup> bone marrow showed no differences in incubation period or disease susceptibility (Mohan *et al.*, 2004). This suggests that stromal cells expressing PrP are important, and, in the light of the FDC study mentioned above (Mohan *et al.*, 2005a), these stromal cells are likely to be FDCs.

After inoculation via skin scarification of the thigh, the scrapie agent first accumulates in the draining inguinal lymph node (ILN) prior to detection in the non-draining lymph nodes and the spleen (Mohan *et al.*, 2005a). However, whether neuroinvasion occurs directly from the draining ILNs, spleen or a combination of lymphoid tissues is not known. In this chapter, mice lacking ILNs were used to investigate the importance of the draining ILN in scrapie pathogenesis after inoculation via scarification of the skin of the thigh.

## **5.3 Results**

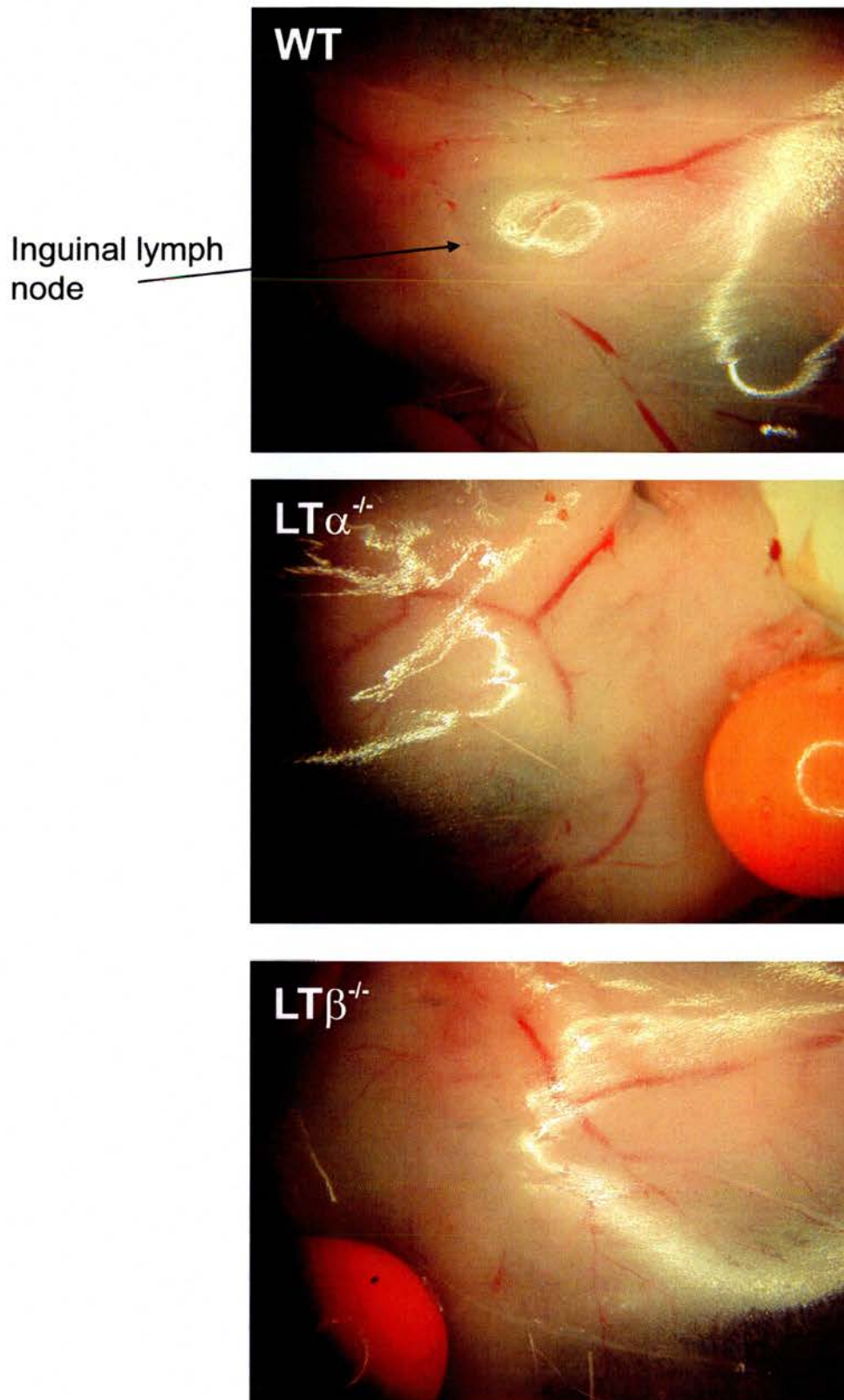
### **5.3.1 Creation of mice that lack ILNs but retain FDCs in remaining lymphoid tissues**

ILNs were absent in lymphotoxin (LT)  $\alpha$  deficient ( $LT\alpha^{-/-}$ ) mice and  $LT\beta^{-/-}$  mice (Fig 5.1), as  $LT\alpha_1\beta_2$  signalling through  $LT\beta$  -receptor ( $LT\beta R$ ) is crucial for the formation of many secondary lymphoid tissues. The formation of ILNs was not induced by the reconstitution of  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice with wild-type (WT) bone marrow (termed WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice respectively; data not shown). However, this treatment induced the terminal differentiation of FDCs and organisation of the microarchitecture in the remaining lymph nodes and spleen of WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice (Fig 4.2). ILNs were also absent from mice which had been treated *in utero* with  $LT\beta R$ -Ig (data not shown). As discussed in section 4.4, these mice have FDCs in the remaining lymphoid tissues as the effect of the treatment on  $LT\alpha_1\beta_2$  signalling is temporary and is restored following catabolism of  $LT\beta R$ -Ig permitting subsequent FDC maturation.

### **5.3.2 Effect of ILN deficiency on scrapie pathogenesis after inoculation via scarification**

WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were  $\gamma$ -irradiated and reconstituted with WT bone marrow (termed WT->WT mice, WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice respectively). 35 days later mice were inoculated with the scrapie agent via scarification of the skin of the right thigh. All WT->WT mice developed disease with a mean incubation period of  $322 \pm 9$  days (Table 5.1). In contrast, only one out of 7 WT-> $LT\alpha^{-/-}$  mice developed disease by the time the experiment was terminated





**Figure 5.1- Presence of inguinal lymph nodes (ILNs) in wild-type (WT) mice and absence of ILNs in lymphotoxin  $\alpha$  deficient (LT $\alpha$ <sup>-/-</sup>) mice and lymphotoxin  $\beta$  deficient (LT $\beta$ <sup>-/-</sup>) mice.** ILNs are clearly visible situated in the subcutaneous fat close to the bifurcation of the superficial epigastric vein of WT mice (upper row). ILNs are absent from the same anatomical location in LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice (middle & lower row).

	Presence at inoculation		Incidence <sup>1</sup>	Mean incubation period
	SPL	ILNs		
WT->WT	+	+	9/9	322 ± 9
WT->LTα <sup>-/-</sup>	+	-	1/7	343, 6 ≤ 503
WT->LTβ <sup>-/-</sup>	+	-	2/8	342 ± 11, 1 ≤ 503 <sup>a</sup>
C57BL/Dk (control)	+	+	5/5	322 ± 10
ILN deficient C57BL/Dk	+	-	3/5	328 ± 15, 2 ≤ 480

**Table 5.1 – Effect of ILN deficiency on scrapie susceptibility after inoculation via scarification of the skin of the thigh.**

1. Incidence = number of animals affected/ number of animals tested.

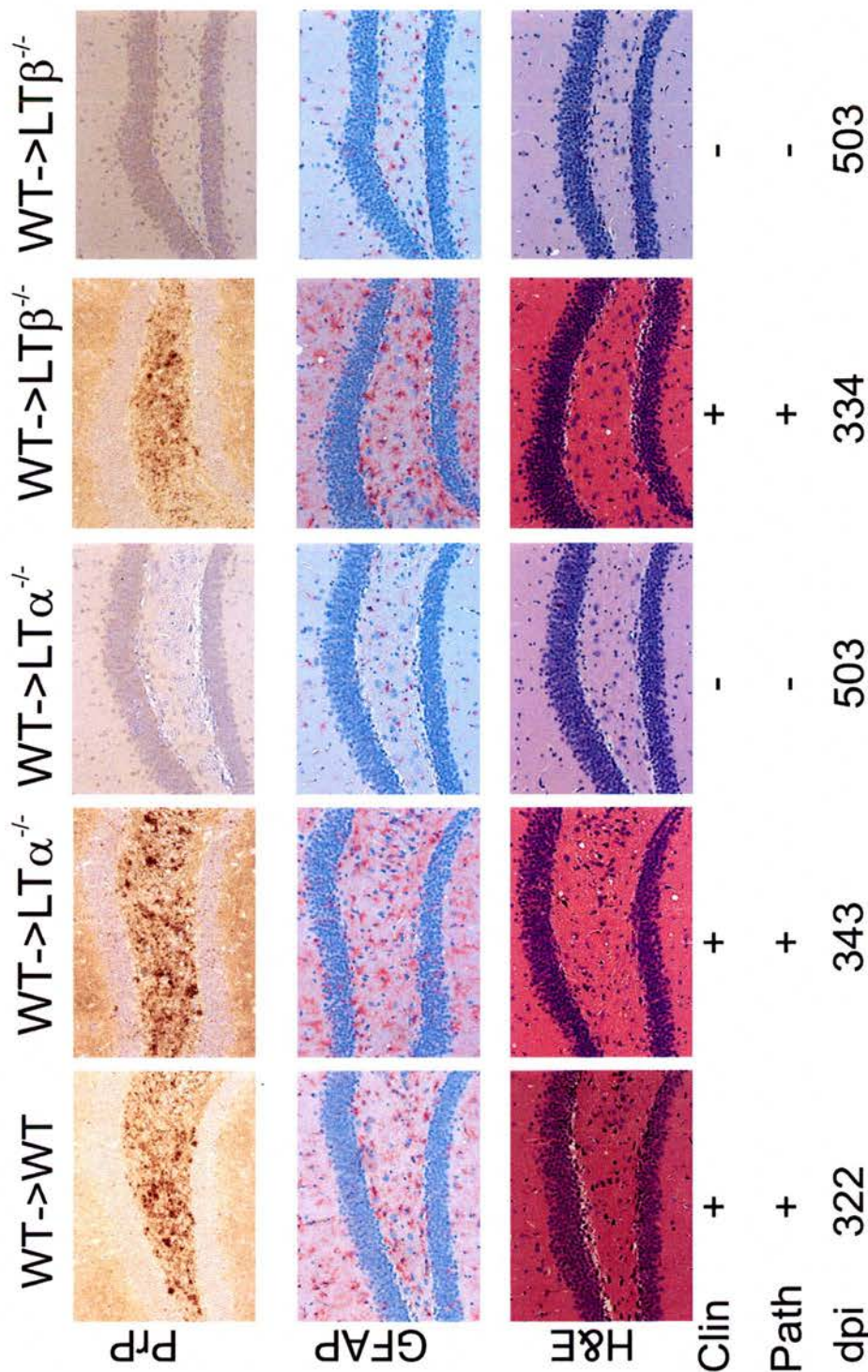
a. Five mice were killed 403, 406, 476 and 490 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull and no histopathological signs of scrapie were detected in the brain (data not shown).

SPL = spleen; ILNs = inguinal lymph nodes; WT->WT, WT->LTα<sup>-/-</sup> and WT->LTβ<sup>-/-</sup> are wild-type mice, lymphotoxin –α deficient mice and lymphotoxin –β deficient mice respectively, reconstituted with wild-type bone marrow.  
+ = presence, - = absence

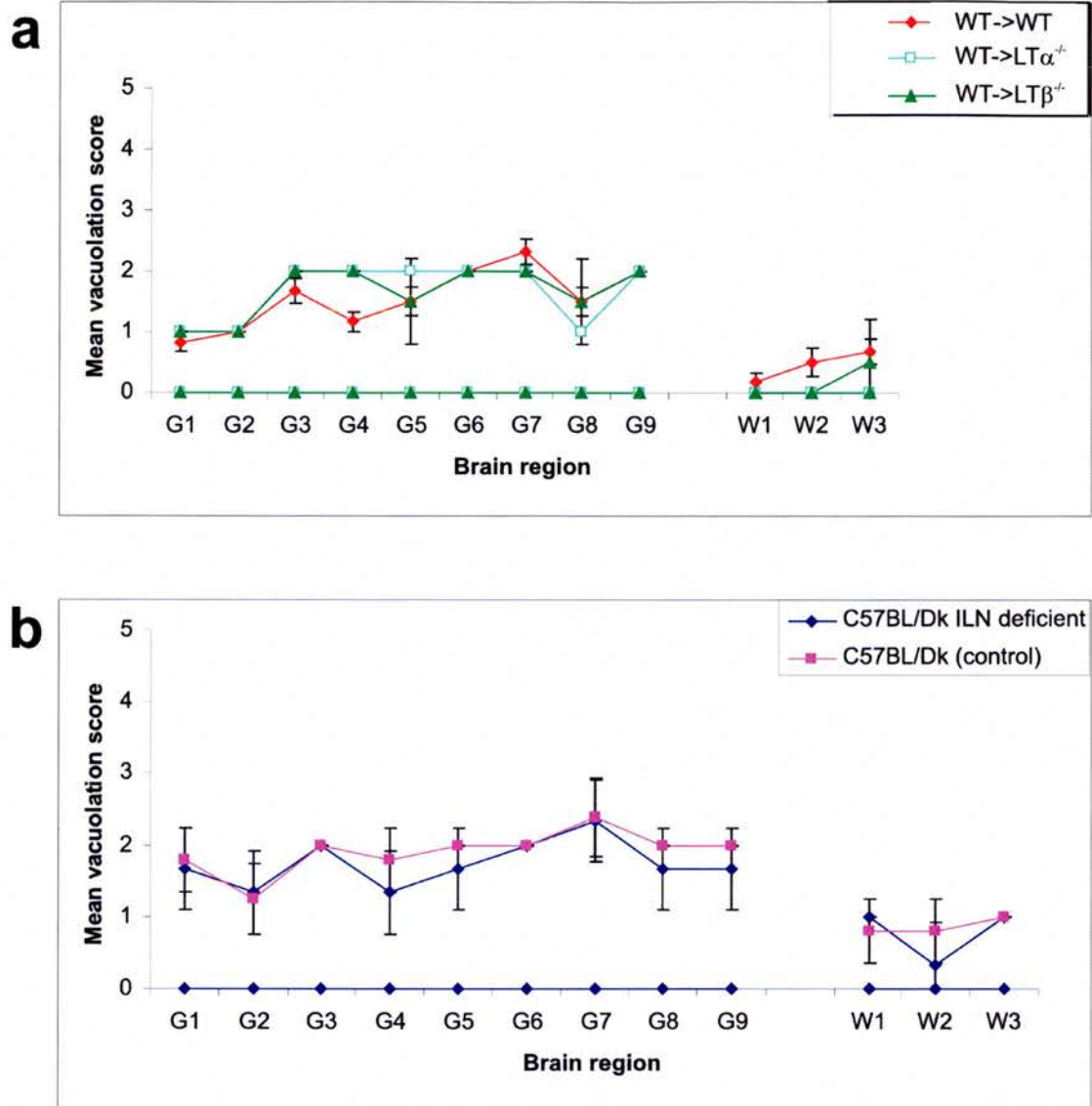
at 503 days post inoculation (dpi). Similarly 2 of seven WT->LT $\beta^{-/-}$  mice developed clinical signs of scrapie, and 5 mice culled between 403 and 490 dpi for ethical reasons were clinically negative. One WT->LT $\beta^{-/-}$  mouse remained free of the clinical and pathological signs of scrapie at the end of the experiment (503 dpi). As discussed in section 1.1.4, two terms are used in this thesis to describe disease specific PrP. Where proteinase K resistance of disease specific PrP is confirmed the term PrP<sup>Sc</sup> is used. Otherwise, disease specific PrP is referred to as PrP<sup>d</sup>. Histopathological analysis revealed characteristic spongiform pathology, PrP<sup>d</sup> accumulation and reactive astrocytes in the brains of all WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice which were scored as having the clinical signs of disease as described in section 2.3.6 (clinically positive; Fig 5.2). The severity and distribution of the pathological targeting of the vacuolation in the brains of WT->WT mice was typical of an infection with the ME7 scrapie agent. No significant differences were observed in the severity and distribution of the pathological targeting of vacuolation in the brains of clinically positive WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice (Fig 5.3). In contrast, no evidence of spongiform pathology, PrP<sup>d</sup> accumulation or reactive astrocytes was detected in the brains of surviving WT->LT $\alpha^{-/-}$  mice or clinically negative WT->LT $\beta^{-/-}$  mice (Fig 5.2).

Mice treated *in utero* with LT $\beta$ R-Ig (ILN-deficient C57BL/Dk mice) and untreated C57BL/Dk mice (immunocompetent controls) were inoculated with the ME7 scrapie agent via scarification at 8-12 weeks of age. All C57BL/Dk (control) mice succumbed to disease with an average incubation period of  $322 \pm 10$  days ( $n = 5$ ; Table 5.1). In contrast, only 3 out of 5 ILN-deficient C57BL/Dk mice succumbed to





**Figure 5.2- Histological analysis of brain tissue from terminally scrapie affected mice or survivor mice.** Large disease specific prion protein (PrP<sup>Sc</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score either positive (+) or negative (-); Path, pathological score either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are wild-type mice, lymphotoxin  $\alpha$ -deficient mice and lymphotoxin  $\beta$ -deficient mice respectively, reconstituted with wild-type bone marrow.



**Figure 5.3- Pathological targeting of vacuolation in the brains of mice terminally affected by ME7 scrapie or survivor mice. a)** wildtype mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice were reconstituted with wildtype bone marrow (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice respectively) and inoculated 35 days later with ME7 scrapie via scarification of the skin. **b)** Inguinal lymph node (ILN)-deficient C57BL/Dk mice and C57BL/Dk (control) mice were inoculated with ME7 scrapie via scarification of the skin. Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (G1-G9) or white matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of the superior peduncles; W3, cerebral peduncles. Each point represents mean vacuolation score  $\pm$  S.E.M. for groups of 6-9 mice.

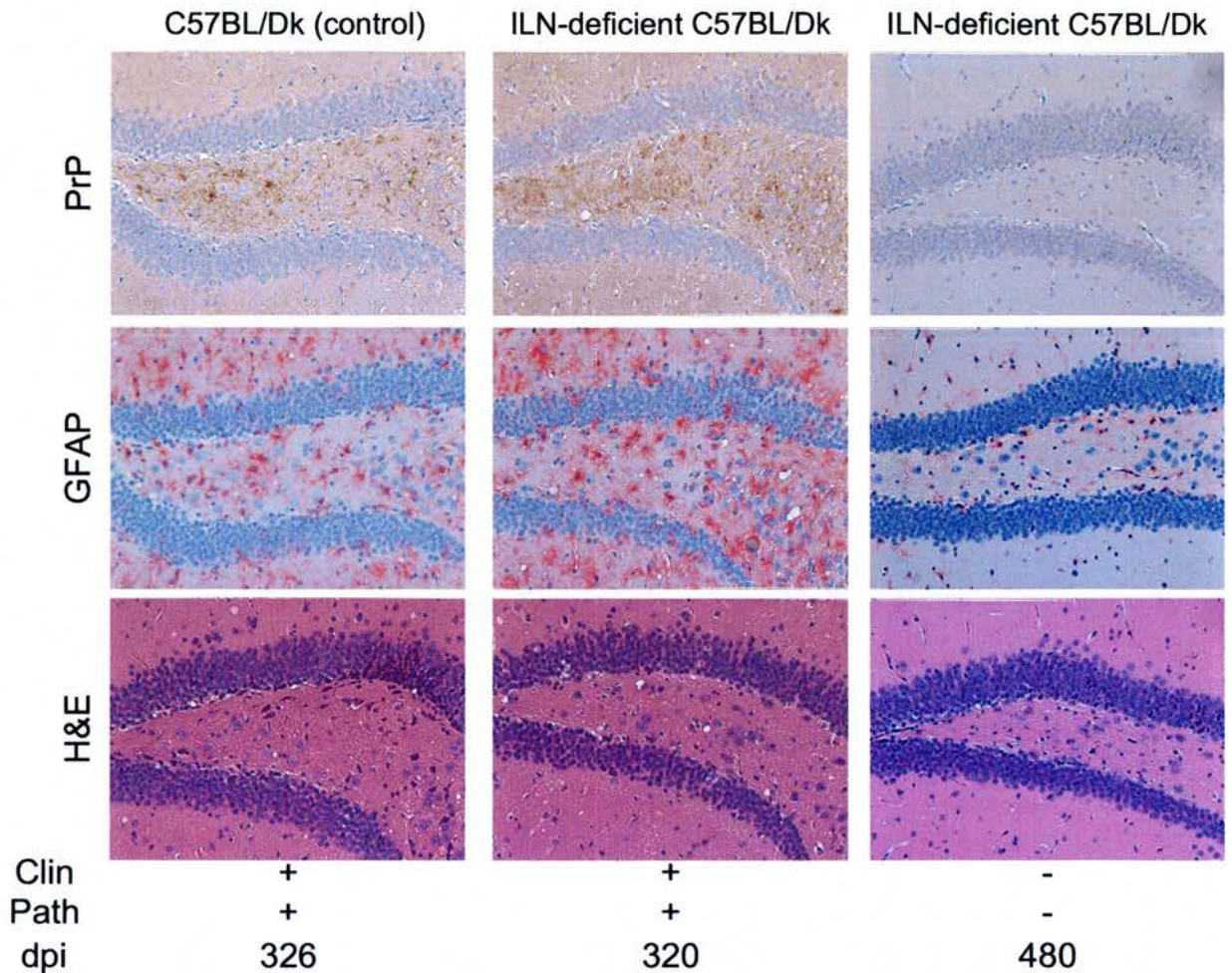


disease in a similar time frame (mean incubation period  $328 \pm 15$  days,  $n = 3$ ). Two ILN-deficient C57BL/Dk mice remained free of the signs of scrapie until at least 480 dpi (Table 5.1). Histopathological analysis of the brain revealed characteristic spongiform pathology, PrP<sup>d</sup> accumulation and reactive astrocytes in the brains of all C57BL/Dk mice and ILN-deficient C57BL/Dk mice which were scored clinically positive (Fig 5.4). The severity and distribution of the pathological targeting of the vacuolation in the brains C57BL/Dk control mice was typical of an infection with the ME7 scrapie agent. No significant differences were observed in the severity and distribution of the pathological targeting of vacuolation in the brains of clinically positive ILN-deficient C57BL/Dk mice (Fig 5.3). In contrast, no evidence of spongiform pathology, PrP<sup>d</sup> accumulation or reactive astrocytes was detected in the brains of the surviving ILN-deficient C57BL/Dk mice (Fig 5.4).

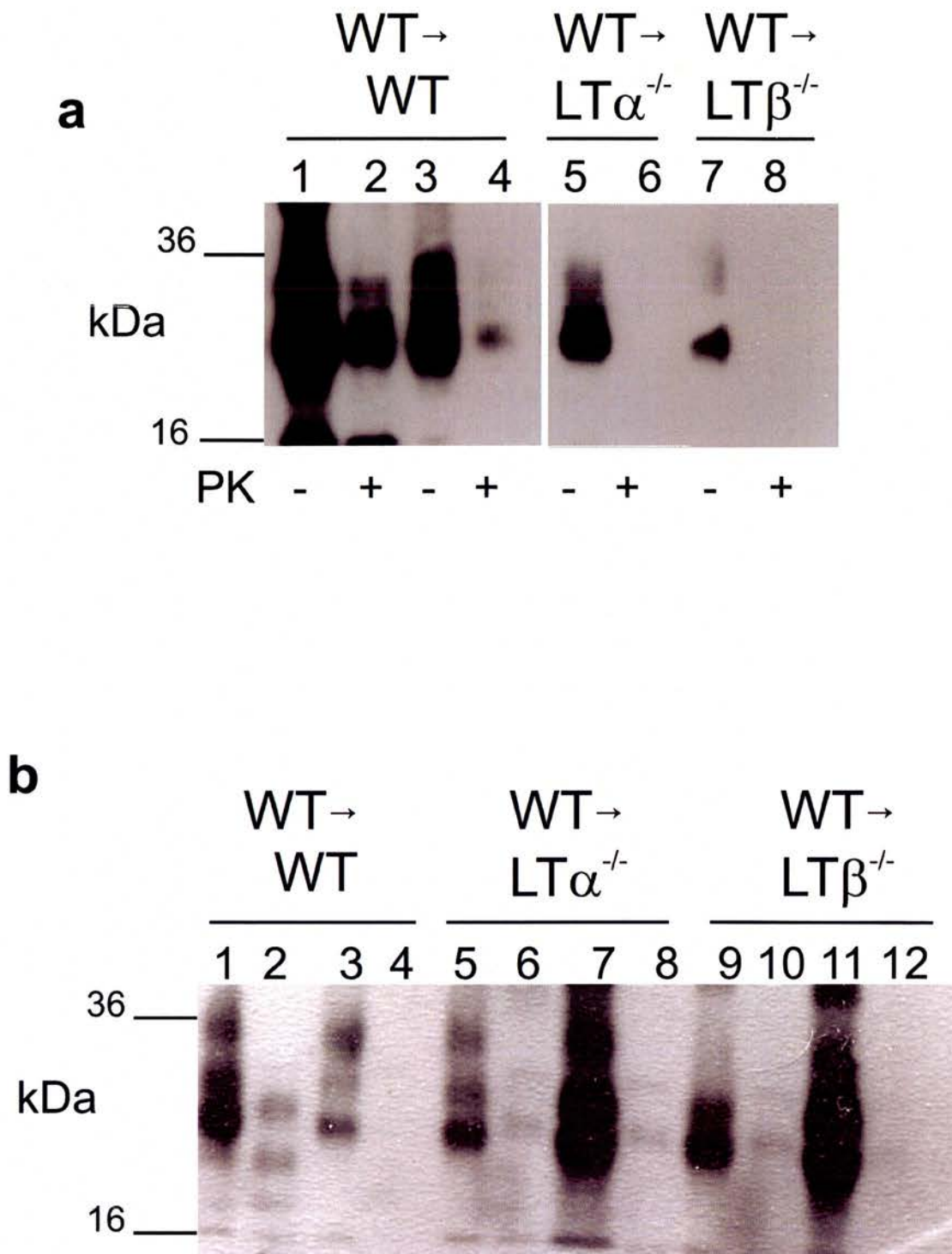
### **5.3.3 Effect of ILN deficiency on the early accumulation of PrP<sup>Sc</sup>**

Within 70 days of inoculation via scarification, the proteinase K (PK) resistant, disease specific isomer of PrP (PrP<sup>Sc</sup>) was detected in ILNs and in a few spleens of WT->WT and C57BL/Dk (control) mice at this time point (Figs 5.5 & 5.6). Tissue from the area where the ILN should have formed in WT->LT $\alpha$ <sup>-/-</sup> mice, WT->LT $\beta$ <sup>-/-</sup> mice and ILN-deficient C57BL/Dk mice was analysed and no PrP<sup>Sc</sup> was detected (Figs 5.5 & 5.6). PrP<sup>Sc</sup> was also absent from the spleens of WT->LT $\alpha$ <sup>-/-</sup> mice, WT->LT $\beta$ <sup>-/-</sup> mice and ILN-deficient C57BL/Dk mice at this time point (Figs 5.5 & 5.6).





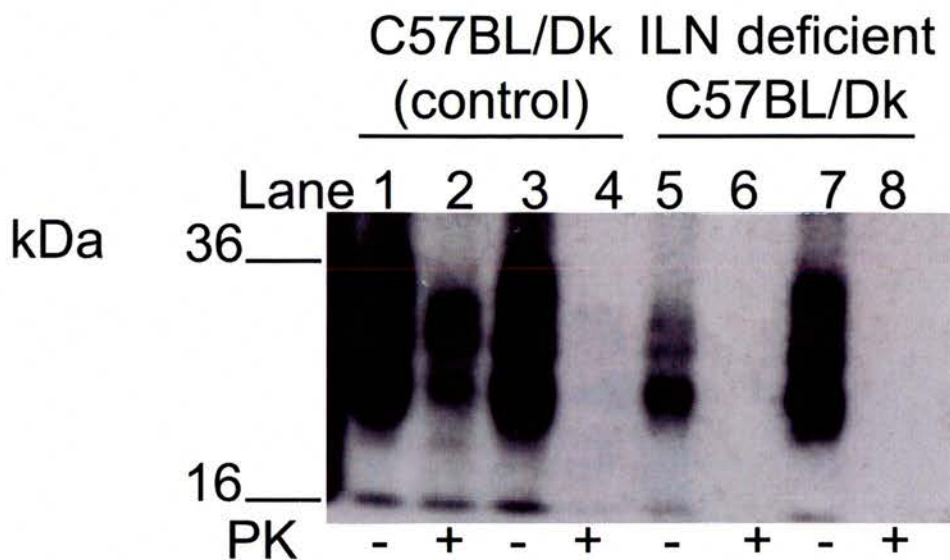
**Figure 5.4- Histological analysis of brain tissue from terminally scrapie affected mice or survivor mice.** Large PrP<sup>d</sup> accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-); Path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis.



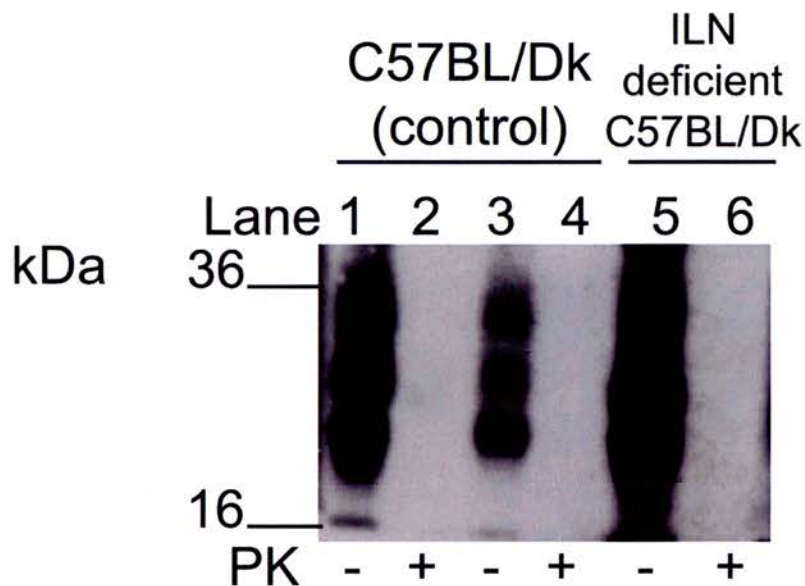
**Figure 5.5- PrP<sup>Sc</sup> accumulation in lymphoid tissues of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin -α deficient mice and lymphotoxin -β deficient mice (WT->WT mice, WT->LTα<sup>-/-</sup> mice and WT->LTβ<sup>-/-</sup> mice respectively) 70 days after inoculation with ME7 scrapie via scarification of the skin. (a) Inguinal lymph nodes (ILNs; or tissue where ILNs should have been in WT->LTα<sup>-/-</sup> mice and WT->LTβ<sup>-/-</sup> mice) and (b) spleens. Proteinase K (PK) resistant disease specific prion protein (PrP<sup>Sc</sup>) was detected in ILNs and some spleens from WT->WT mice. No PrP<sup>Sc</sup> was detected in tissues where ILNs should have been or spleens from either WT->LTα<sup>-/-</sup> mice or WT->LTβ<sup>-/-</sup> mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis.**



**a**



**b**

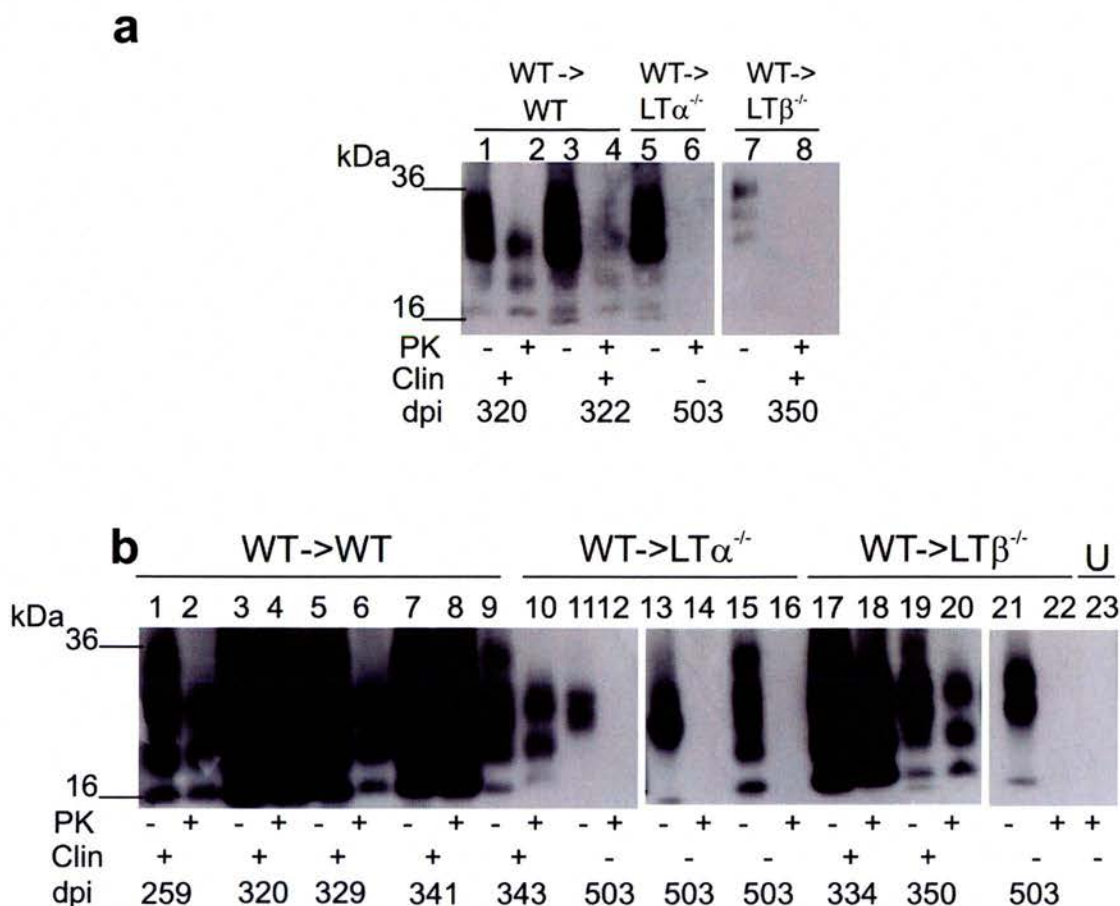


**Figure 5.6- PrP<sup>Sc</sup> accumulation in lymphoid tissues in ILN-deficient C57BL/Dk and C57BL/Dk (control) mice 70 days after inoculation via scarification of the skin. (a)** Inguinal lymph nodes (ILNs; or tissue where ILNs should have been in ILN-deficient C57BL/Dk mice) and **(b)** spleens. Proteinase K resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in some ILNs from control mice. No PrP<sup>Sc</sup> was detected in tissues where ILNs should have been in ILN-deficient C57BL/Dk mice or spleens from either control or ILN-deficient C57BL/Dk mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis.

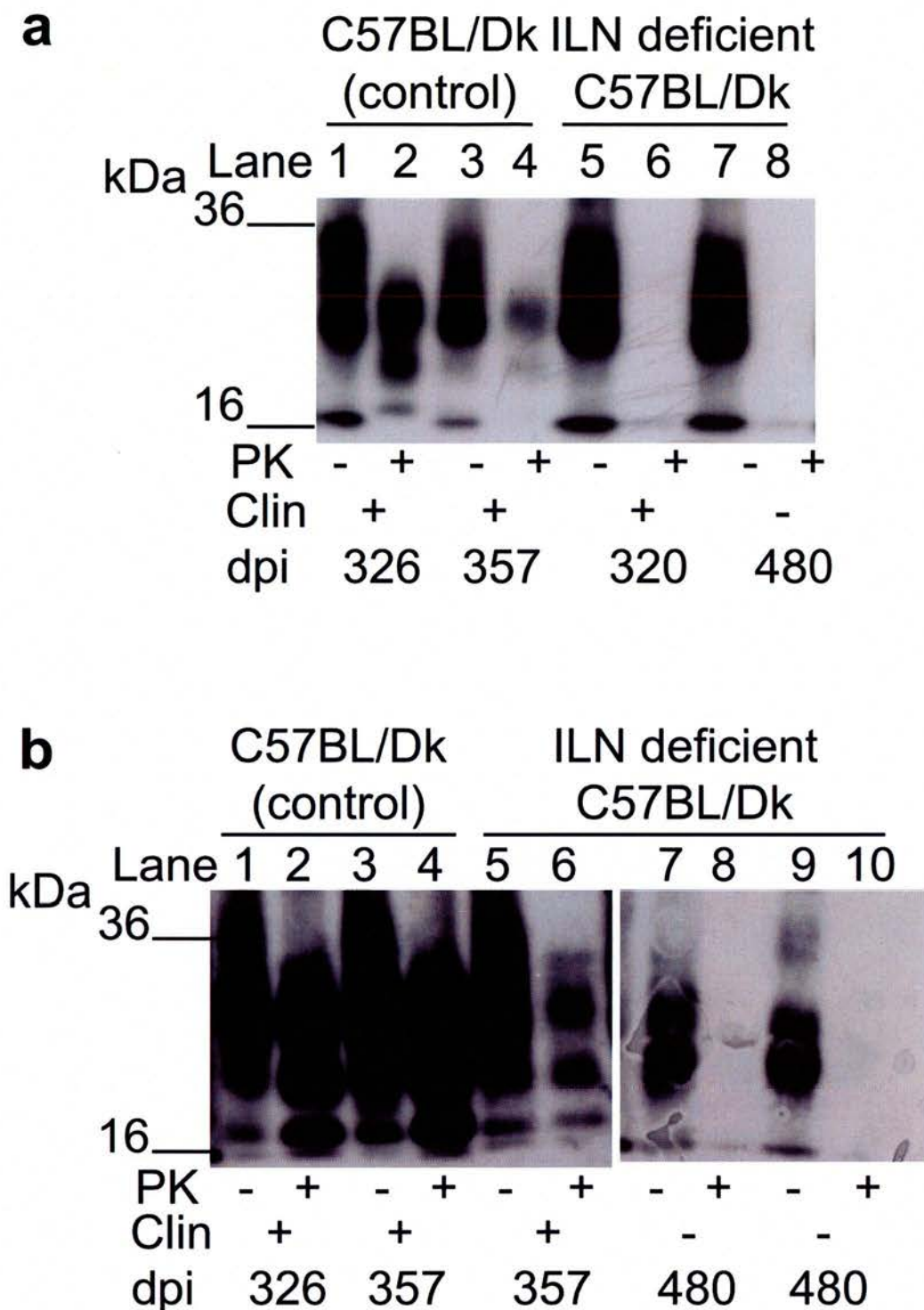


#### **5.3.4 Effect of ILN deficiency on the late accumulation of PrP<sup>Sc</sup>**

At the terminal stage of disease high levels of PrP<sup>Sc</sup> are found in the ILNs and spleens of immunocompetent mice (Mohan *et al.*, 2004). Consistent with this, PrP<sup>Sc</sup> was detected in ILNs and spleens of all WT->WT and C57BL/Dk (control) mice at the end stage of disease (Figs 5.7 & 5.8). Tissue from the area where the ILN should have formed in WT->LT $\alpha$ <sup>-/-</sup> mice, WT->LT $\beta$ <sup>-/-</sup> mice and ILN-deficient C57BL/Dk mice was analysed and no PrP<sup>Sc</sup> was detected in either clinically positive or clinically negative cases (Figs 5.7 & 5.8). Spleens from all clinically positive WT->LT $\alpha$ <sup>-/-</sup> mice, WT->LT $\beta$ <sup>-/-</sup> mice and ILN-deficient C57BL/Dk mice were found to contain high levels of PrP<sup>Sc</sup> (Figs 5.7 & 5.8) In contrast, no PrP<sup>Sc</sup> was detected in the spleens of survivor WT->LT $\alpha$ <sup>-/-</sup> mice, WT->LT $\beta$ <sup>-/-</sup> mice or ILN-deficient C57BL/Dk mice (Figs 5.7 & 5.8).



**Figure 5.7- PrP<sup>Sc</sup> accumulation in lymphoid tissues of wild-type (WT) bone marrow reconstituted WT mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice (WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice, respectively). (a) Inguinal lymph nodes (ILNs; or tissue where ILNs should have been in WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice) and (b) spleens of clinically affected WT->WT mice, WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice, or survivor WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice after inoculation with ME7 scrapie via scarification of the skin. Proteinase K (PK) resistant disease specific prion protein (PrP<sup>Sc</sup>) was detected in the ILNs and the spleens of WT->WT mice and the spleens of terminally scrapie affected WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  mice. No PrP<sup>Sc</sup> was detected in tissues where ILNs should have been in WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  or spleens from survivor WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice or from mice which were not inoculated with the scrapie agent. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score, either positive (+) or negative (-); dpi, days post inoculation on which the tissues were taken for analysis.**



**Figure 5.8- PrP<sup>Sc</sup> accumulation in lymphoid tissues.** (a) Inguinal lymph nodes (ILNs; or tissue where ILNs should have been in ILN-deficient C57BL/Dk mice) and (b) spleens of clinically affected C57BL/Dk (control) mice and ILN-deficient C57BL/Dk mice after inoculation with ME7 scrapie via scarification of the skin. Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in the ILNs and spleens of clinically affected control mice and in the spleens of clinically affected ILN-deficient mice. No PrP<sup>Sc</sup> was detected in the tissue where the ILN should have been in the ILN deficient mice or in the spleens of clinically negative ILN deficient mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score, either positive (+) or negative (-); dpi, days post inoculation on which the tissues were taken for analysis.



## **5.4 Discussion**

Mice lacking ILNs were inoculated with ME7 scrapie via scarification of the skin of the thigh to investigate the role of the draining lymph nodes in scrapie pathogenesis.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were reconstituted with WT bone marrow to create mice which lacked ILNs but possessed FDCs in remaining lymphoid tissues. ILN-deficient mice were also created by *in utero* treatment with  $LT\beta$ R-Ig. All groups of ILN deficient mice displayed decreased susceptibility to scrapie after inoculation with the ME7 scrapie agent via scarification.

As discussed in section 3.4, the development of most secondary lymphoid tissue is critically dependent upon  $LT\alpha_1\beta_2$  signalling through  $LT\beta$ R during gestation. As a consequence, ILNs do not form in mice where this signalling has been interrupted during gestation, either by genetic deficiency of  $LT\alpha$  (Banks *et al.*, 1995; Togni *et al.*, 1994) or  $LT\beta$  (Koni *et al.*, 1997), or due to pharmacological  $LT\beta$ R-Ig blockade (Rennert *et al.*, 1996). As discussed in section 4.4 the remaining lymphoid tissues of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack FDCs and display disorganised microarchitecture.  $\gamma$ -irradiation and reconstitution of these mice with WT bone marrow induces the development of FDC networks and organisation of lymphoid tissue microarchitecture without inducing the formation of the missing lymphoid tissues (section 4.4). FDCs are present and lymphoid tissue microarchitecture is normal in mice treated *in utero* with  $LT\beta$ R-Ig, as in this case the blockade of  $LT\alpha_1\beta_2$  signalling is temporary and is restored by the time FDCs begin to form (Rennert *et al.*, 1996).

Previous work has shown that a functional immune system is of paramount importance for the development of scrapie disease after inoculation via scarification as SCID mice do not develop disease when inoculated in this way (Taylor *et al.*, 1996b). The important role of FDCs in this process has been shown by the de-differentiation of FDCs (by treatment of adult mice with LT $\beta$ R-Ig) prior to inoculation which decreased the incidence and increased the incubation period of disease (Mohan *et al.*, 2005a). The results of these two studies also demonstrate an important role for another immune system component, independent of FDCs, as some cases of scrapie disease were able to occur in the absence of FDCs (Mohan *et al.*, 2005a) whereas none were able to occur in the absence of a functioning immune system (Taylor *et al.*, 1996b).

Treatment of adult mice with LT $\beta$ R-Ig as described above (Mohan *et al.*, 2005a) de-differentiates FDCs in all lymphoid tissues. Here, by using mice that lack the draining ILNs, the effects of a lack of FDCs local to the site of inoculation could be determined. In these mice a decrease in the incidence of disease was observed demonstrating the importance of the draining lymphoid tissues for the development of disease. However, some mice did succumb to disease, most likely following neuroinvasion from other lymphoid tissues as the remaining lymphoid tissues of these mice contained FDCs. The time of onset of disease in mice lacking ILNs did not differ significantly from control mice demonstrating that, in a few cases, non draining lymphoid tissue can support neuroinvasion as effectively as the draining ILNs.

The function of the draining ILN in scrapie pathogenesis after inoculation via scarification may be to support neuroinvasion after accumulation of the agent on local FDCs. In the absence of the draining ILN the scrapie agent may, in a few cases, be able travel to other lymphoid tissues from which neuroinvasion can occur. It could be that in some cases in the absence of the draining ILN, the scrapie agent enters the draining lymph and becomes so disseminated that the agent is cleared, perhaps by macrophages (Carp & Callaghan, 1982) or dendritic cells (Mohan *et al.*, 2005c). However, in a few cases a small amount of the agent reaches other lymphoid tissues from which neuroinvasion can occur. Alternatively, it could be that neuroinvasion does not occur directly from the draining ILNs and instead the draining ILN propagates the scrapie agent sufficiently to ensure the spread to other lymphoid tissues prior to neuroinvasion. Thus in the absence of this initial amplification stage, the level of the scrapie agent reaching more distant lymphoid tissues is variable and sufficient only for the establishment of disease in some cases. There is also the possibility that, particularly in the case of the one clinically positive WT->LT $\alpha^{-/-}$  mouse, that there could have been direct inoculation of a small amount of infectivity into bloodstream or into a nerve at the site of inoculation thus bypassing the need for any replication of the scrapie agent in the draining lymphoid tissues.

The lymphoid tissues remaining in WT->LT $\alpha^{-/-}$  mice consist of spleen and nasal associated lymphoid tissue (NALT; Banks *et al.*, 1995; Fukuyama *et al.*, 2002; Togni *et al.*, 1994). WT->LT $\beta^{-/-}$  mice, as well as containing spleen and NALT, also have mesenteric lymph nodes (MLNs) and cervical lymph nodes (CLNs; Fukuyama *et al.*,



2002; Koni *et al.*, 1997). The remaining lymphoid tissues in mice treated *in utero* with LT $\beta$ R-Ig on day 11.5 of gestation consist of spleen, NALT, MLNs, CLNs and also lumbar and sacral lymph nodes (Rennert *et al.*, 1997; Rennert *et al.*, 1996). It is possible that some of these remaining lymphoid tissues may drain the skin but it is unlikely that they would drain the skin of the thigh due to their anatomical location. However, these lymphoid tissues may accumulate the scrapie agent once the draining lymph has passed through the thoracic duct into the bloodstream. Data presented here showed that WT->LT $\alpha^{-/-}$  mice have a slightly lower incidence of scrapie compared to WT->LT $\beta^{-/-}$  mice, and WT->LT $\beta^{-/-}$  mice had a lower incidence compared to *in utero* LT $\beta$ R-Ig treated mice. Thus in mice with more remaining lymphoid tissues there was a higher incidence of scrapie after inoculation via scarification. This could be because there would be a higher chance that the scrapie agent would reach the remaining lymphoid tissues in mice where more are present.

Data presented here showed that lymphoid tissue local to the site of inoculation was important for the successful development of clinical disease after inoculation with ME7 scrapie via scarification of the skin of the thigh. Neuroinvasion was able to occur from more distant lymphoid tissue in some cases, demonstrating that other lymphoid tissues were able to compensate for the absence of the local lymphoid tissues, but this route is extremely inefficient.

## Role of the draining lymph nodes in scrapie pathogenesis after inoculation via intra-peritoneal injection

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## **6.1 Abstract**

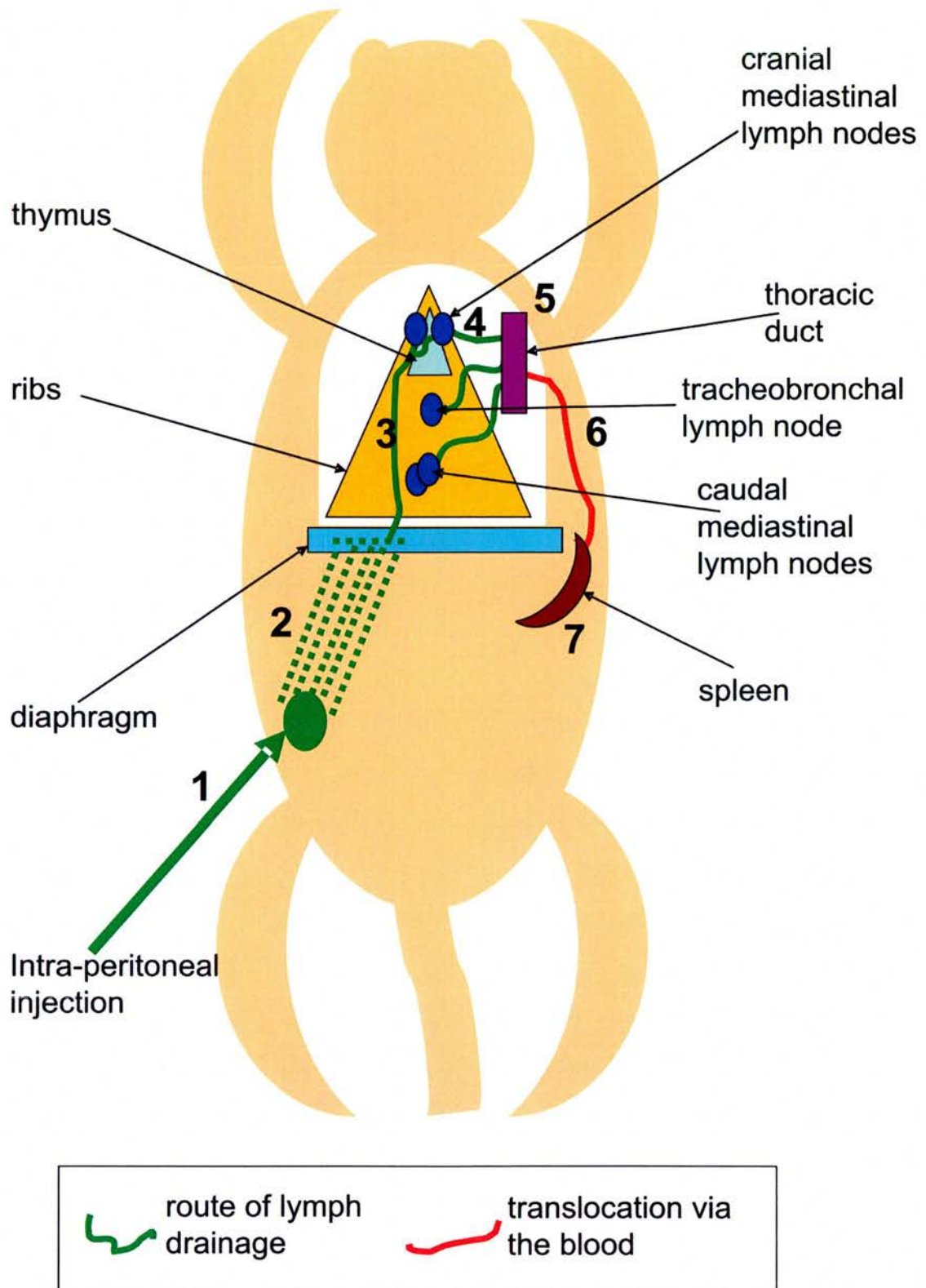
Inoculation via intra-peritoneal (i.p.) injection is a commonly used route for the investigation of the peripheral pathogenesis of TSEs. The peritoneal cavity is thought to be drained by the cranial mediastinal lymph nodes (CMLNs). Mice lacking the CMLNs were inoculated with the ME7 strain of the scrapie agent via i.p. injection to determine the importance of draining lymphoid tissues in scrapie pathogenesis. Lymphotoxin (LT)- $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice lack the CMLNs and lack follicular dendritic cells (FDCs) in the remaining lymphoid tissues. These mice and wild-type (WT) control mice were inoculated with the ME7 strain of the scrapie agent via i.p. injection to determine the importance of both lymphoid tissues and FDCs on scrapie pathogenesis. LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice exhibited reduced susceptibility and delayed onset of disease compared to WT mice. These data are consistent with previous studies demonstrating that neuroinvasion after i.p. inoculation is less efficient in the absence of both the local lymph nodes and FDCs in remaining lymphoid tissues. Next, LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice were reconstituted with WT bone marrow (termed WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice respectively) to restore FDCs to the remaining lymphoid tissues without inducing the formation of the missing lymphoid tissues. WT->LT $\alpha^{-/-}$  mice displayed reduced susceptibility and delayed onset of disease, whereas WT->LT $\beta^{-/-}$  mice were as susceptible and developed disease in the same time frame as WT->WT control mice. These data demonstrate that the CMLNs are not important in scrapie pathogenesis and raised the possibility that another lymphoid tissue present in WT->LT $\beta^{-/-}$  mice, but absent from WT->LT $\alpha^{-/-}$  mice, was playing an important role in scrapie pathogenesis. These data also demonstrate the importance of restoring the differentiation of FDCs in studies



using  $LT\alpha^{-/-}$  mice, as both  $LT\alpha^{-/-}$  mice and WT- $\rightarrow$  $LT\alpha^{-/-}$  mice displayed decreased susceptibility to i.p. inoculated scrapie agent.

## **6.2 Introduction**

Although the pathology caused by TSEs occurs predominantly within the central nervous system (CNS), considerable effort has been put into determining the peripheral course of the disease. As neurodegeneration is not at present pharmacologically reversible, intervention targets in the periphery prior to the onset of neurodegeneration are desirable. Much of the work on the peripheral pathogenesis of TSEs has involved the experimental inoculation of rodents via the intra-peritoneal (i.p.) route. Although the i.p. route does not directly model the routes postulated for the establishment of natural TSE disease, the ease of the inoculation procedure and certainty with which all animals can be assumed to have been dosed equally make this route attractive. The draining lymphoid tissue after i.p. inoculation is commonly perceived to be the spleen. However, studies have shown that the diaphragm initially drains the peritoneum (Shinohara, 1997), lymph from the peritoneum then drains towards the lymph nodes within the thoracic cavity. There is some confusion about the nomenclature of the lymph nodes draining the peritoneal cavity. These have been referred to as parathymic lymph nodes (Hill, 1976) and also mediastinal lymph nodes (Marco *et al.*, 1992). A recent paper has addressed the issue of lymph node nomenclature and has termed both of these as cranial mediastinal lymph nodes (CMLNs; Broeck *et al.*, 2006). In this thesis the lymph nodes will therefore be described as CMLNs. From the CMLNs lymph enters the bloodstream via the thoracic duct and disseminates systemically, thus reaching other lymphoid tissues such as the spleen (Fig 6.1).



**Figure 6.1- Proposed model of lymphatic drainage of the peritoneal cavity.**  
**1.** Injection of antigens or particles into the upper flank. **2.** Peritoneal fluid drains towards the diaphragm. **3.** From the diaphragm lymph travels in lymph vessels towards the cranial mediastinal lymph nodes. **4.** Efferent lymphatics from these lymph nodes connect directly with the thoracic duct. **5.** Lymph is returned to the blood via the thoracic duct. **6.** Antigens or particles from the peritoneal cavity are then widely distributed via the blood-stream and may be sequestered in other lymphoid tissues including the spleen (**7**).



The involvement of lymphoid tissues in TSE pathogenesis via i.p. inoculation was initially demonstrated by the extended incubation period observed in mice which had been splenectomised prior to or slightly after inoculation (Fraser & Dickinson, 1978). Follicular dendritic cells (FDCs) within lymphoid tissues have since been shown to be involved in the pathogenesis of TSEs after i.p. inoculation. Mice lacking FDCs either through genetic deficiency of tumour necrosis factor (TNF)- $\alpha$  (Mabbott *et al.*, 2000b; Prinz *et al.*, 2002), lymphotoxin (LT)- $\alpha$  (Oldstone *et al.*, 2002; Prinz *et al.*, 2002) or LT $\beta$  (Manuelidis *et al.*, 2000; Oldstone *et al.*, 2002; Prinz *et al.*, 2002), or temporary depletion of FDCs at the time of inoculation by treatment with LT $\beta$ R-Ig (Mabbott *et al.*, 2000a; Mabbott *et al.*, 2003; Montrasio *et al.*, 2000) or TNFR-Ig (Mabbott *et al.*, 2002) show decreased susceptibility to disease. How the TSE agent is transported from the site of inoculation to the lymphoid tissues is unclear. This may involve uptake by dendritic cells (Huang *et al.*, 2002; Kaneider *et al.*, 2003) or macrophages (Beringue *et al.*, 2000; Carp & Callaghan, 1982; Jeffrey *et al.*, 2000) or may involve a cell free pathway.

Although it is clear from the work described above that lymphoid tissues are involved in TSE pathogenesis, from which lymphoid tissues neuroinvasion occurs after i.p. inoculation with the scrapie agent is uncertain. Mice lacking particular sets of lymphoid tissues were used in this chapter to determine the importance of lymphoid tissue draining the peritoneal cavity in disease pathogenesis after inoculation with the scrapie agent via i.p. injection.

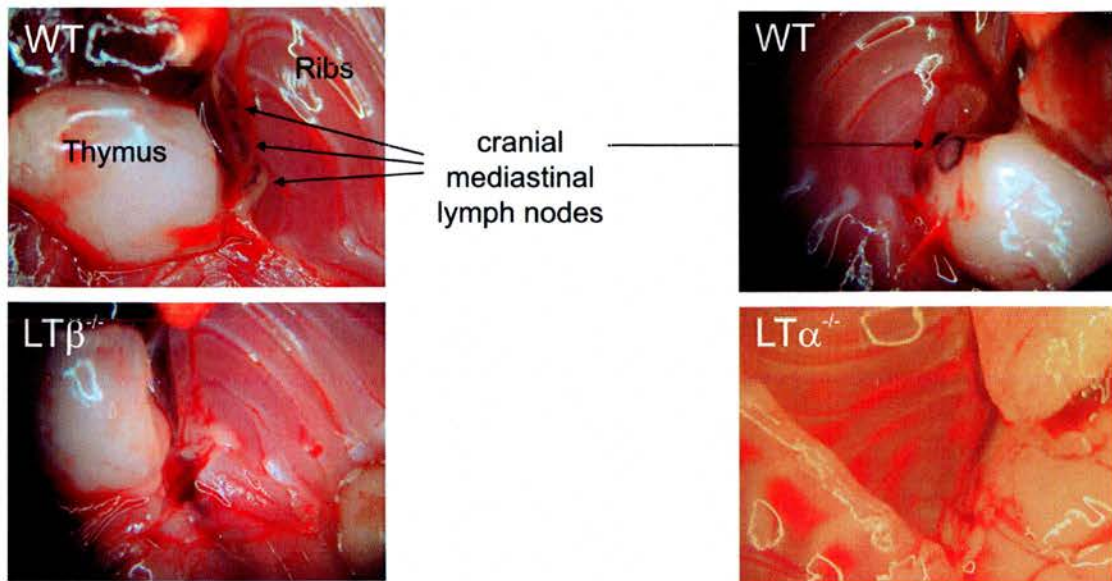
## **6.3 Results**

### **6.3.1 $LT\alpha^{-/-}$ mice and $LT\beta^{-/-}$ mice lack CMLNs**

Wild-type (WT) mice,  $LT\alpha^{-/-}$  mice, and  $LT\beta^{-/-}$  mice were inoculated intraperitoneally with Indian ink to visualise the lymph nodes draining the peritoneal cavity. CMLNs stained blue-black with Indian ink were clearly visible around the thymus in WT mice and clearly absent from both  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice (Fig 6.2).

### **6.3.2 Effect of LT deficiency on scrapie pathogenesis following intra-cranial inoculation**

$LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and WT mice were inoculated with either a 1.0 % or 0.01 % dilution of ME7 scrapie brain homogenate via intra-cranial injection. No significant differences were observed in the mean incubation periods of disease in WT mice,  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice when inoculated intra-cranially with 1.0 % scrapie brain homogenate. All mice developed clinical disease between 154 and 182 days after inoculation (Table 6.1). Similarly, no significant differences in the incubation periods of disease were observed between WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice after intra-cranial inoculation with 0.01 % dilution of scrapie brain homogenate. All mice developed clinical disease between 192 and 281 days after inoculation (Table 6.1). This demonstrates that, following inoculation with either moderate (1.0 %) or limiting doses (0.01 %) of the scrapie agent, genetic deficiency of either  $LT\alpha$  or  $LT\beta$  had no effect on the pathogenesis of ME7 scrapie directly within the CNS. Therefore, any effects observed in  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice after peripheral



**Figure 6.2- Effect of lymphotoxin deficiency on the development of cranial mediastinal lymph nodes (CMLNs).** Mice were inoculated intra-peritoneally with Indian ink (blue-black staining). The thoracic cavity was examined 4 hours later. CMLNs were present in wild-type (WT) mice (upper row) and absent from lymphotoxin  $\beta$  deficient ( $LT\beta^{-/-}$ ) mice (lower left) and lymphotoxin  $\alpha$  deficient ( $LT\alpha^{-/-}$ ) mice (lower right).



	1.0 % dilution brain homogenate		0.01 % dilution brain homogenate	
	Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.	Incidence <sup>1</sup>	Mean incubation period (days) ± S.E.M.
WT	6/6	171 ± 4	6/6	212 ± 4
LTα <sup>-/-</sup>	5/5	175 ± 4	6/6	216 ± 13
LTβ <sup>-/-</sup>	5/5	175 ± 1	6/6	204 ± 4

**Table 6.1- Effect of lymphotoxin deficiency on scrapie susceptibility after intracranial inoculation**

1. Incidence = number of animals affected/ number of animals tested.

WT = wild-type mice; LTα<sup>-/-</sup> = lymphotoxin -α deficient mice; LTβ<sup>-/-</sup> = lymphotoxin -β deficient mice.

inoculation could be attributed to an altered efficiency of translocation of the scrapie agent to the CNS.

### **6.3.3 Effect of LT deficiency on scrapie pathogenesis after i.p. inoculation**

Next, WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were inoculated with either a 1.0 % or 0.01 % dilution of ME7 scrapie brain homogenate via i.p. injection. At the lower dose (0.01 %), 10 out of 11 WT mice developed scrapie disease with a mean incubation period of  $339 \pm 4$  days. In contrast, disease susceptibility of LT-deficient mice was dramatically reduced as none of the  $LT\alpha^{-/-}$  mice and only one of 8  $LT\beta^{-/-}$  mice developed clinical scrapie by the time the experiment was terminated at 545 days post inoculation (dpi; Table 6.2). At the higher dose all the WT mice developed disease with a mean incubation period of  $288 \pm 6$  days (Table 6.2). In contrast, 11 out of 15  $LT\alpha^{-/-}$  mice and 15 of 16  $LT\beta^{-/-}$  mice developed scrapie disease by the time the experiment was terminated at 545 dpi. Disease incubation periods were longer and much more variable in the LT-deficient mice (217 to 482 days for  $LT\alpha^{-/-}$  mice; and 226 to 502 days for  $LT\beta^{-/-}$  mice; Table 6.2). As discussed in section 1.1.4, two terms are used in this thesis to describe disease specific PrP. Where proteinase K resistance of disease specific PrP is confirmed the term  $PrP^{Sc}$  is used. Otherwise, disease specific PrP is referred to as  $PrP^d$ . Histopathological analysis of the brain revealed characteristic spongiform pathology,  $PrP^d$  accumulation and reactive astrocytes in the brains of all WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice which were scored as having the clinical signs of disease as described in section 2.3.6 (clinically positive; Figs 6.3 & 6.4). The severity and distribution of the pathological targeting

	Dose	Presence at inoculation			Incidence <sup>1</sup>	Mean incubation period
		SPL	CMLNs	FDCs		
WT	10 <sup>-2</sup>	+	+	+	10/10	288 ± 6
LTα <sup>-/-</sup>	10 <sup>-2</sup>	+	-	-	11/15	329 ± 20, 4 ≤ 545
LTβ <sup>-/-</sup>	10 <sup>-2</sup>	+	-	-	15/16	327 ± 16, 1 ≤ 545
WT	10 <sup>-4</sup>	+	+	+	10/11	339 ± 4, 1 ≤ 545
LTα <sup>-/-</sup>	10 <sup>-4</sup>	+	-	-	0/15	15 ≤ 545
LTβ <sup>-/-</sup>	10 <sup>-4</sup>	+	-	-	1/8	490, 7 ≤ 545

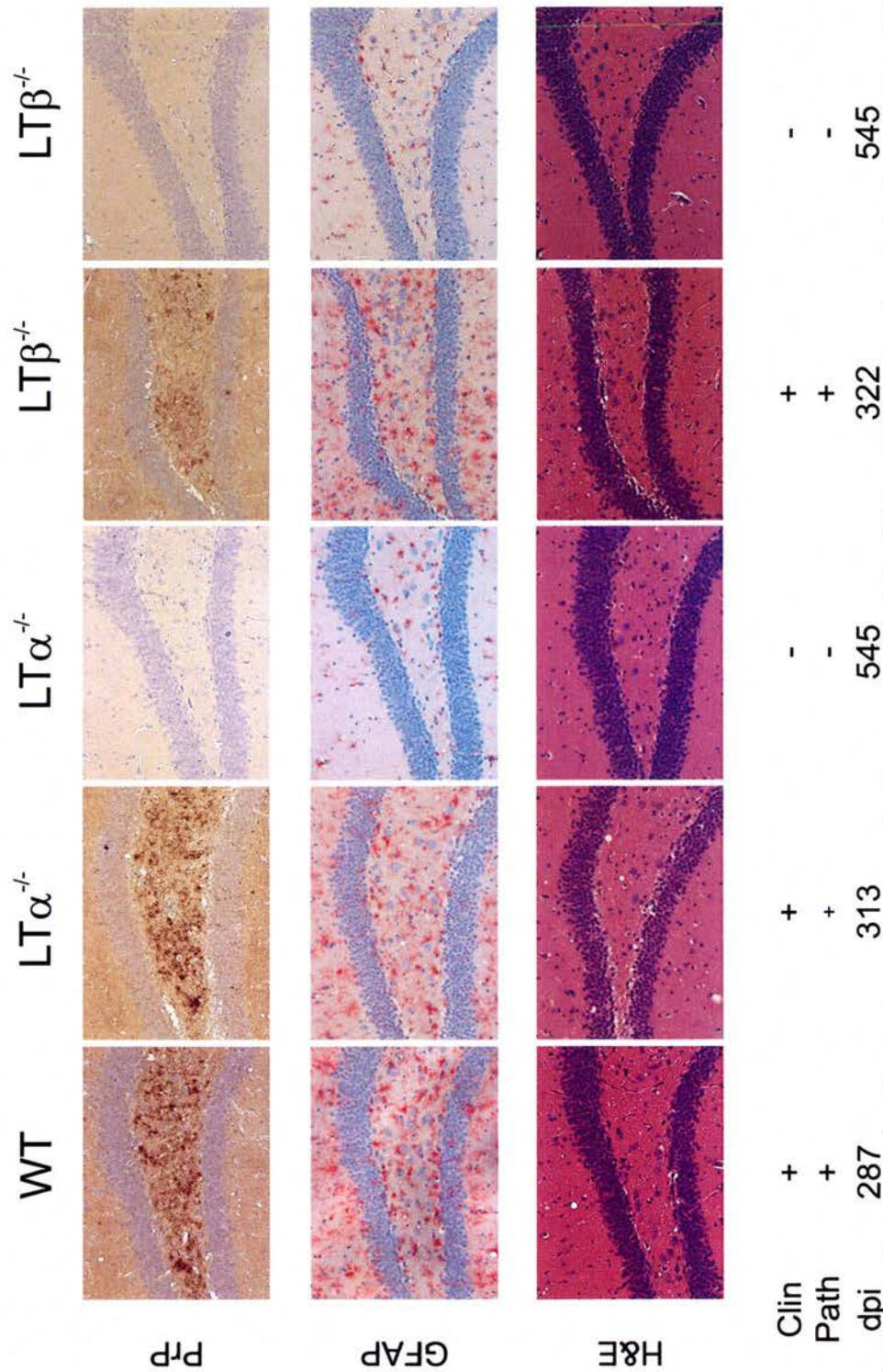
**Table 6.2 – Effect of lymphotoxin deficiency on scrapie susceptibility after inoculation via intra-peritoneal injection.**

1. Incidence = number of animals affected/ number of animals tested.

SPL = Spleen; CMLNs = cranial mediastinal lymph nodes; WT = wild-type mice; LTα<sup>-/-</sup> = lymphotoxin –α deficient mice; LTβ<sup>-/-</sup> = lymphotoxin –β deficient mice.

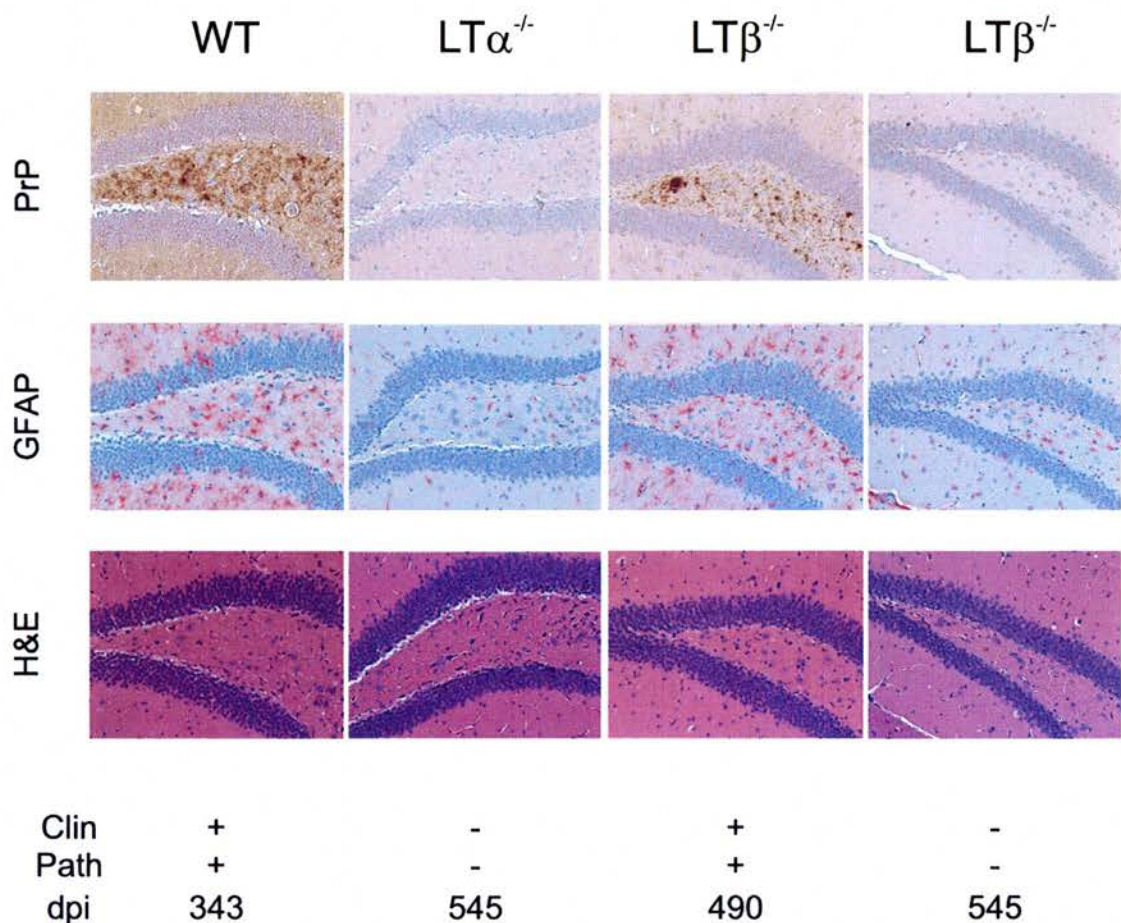
+ = presence, - = absence





**Figure 6.3- Histological analysis of brain tissue from terminally scrapie affected wild-type (WT) mice, lymphotoxin -α deficient (LTα<sup>-/-</sup>) mice or lymphotoxin -β deficient (LTβ<sup>-/-</sup>) mice; or survivor LTα<sup>-/-</sup> mice or LTβ<sup>-/-</sup> mice inoculated intra-peritoneally with 0.01 % dilution of ME7 scrapie brain homogenate.** Large disease specific prion protein (PrP<sup>Sc</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row). Extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-); Path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis.





**Figure 6.4- Histological analysis of brain tissue from terminally scrapie affected wild-type (WT) mice or lymphotoxin  $\beta$  deficient (LT $\beta^{-/-}$ ) mice; or survivor lymphotoxin  $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice or LT $\beta^{-/-}$  mice inoculated intraperitoneally with 1.0 % dilution of ME7 scrapie brain homogenate.** Large disease specific prion protein (PrP<sup>d</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-), Path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis.

of the vacuolation in the brains of WT mice was typical of ME7 scrapie. No significant differences were observed in the severity and distribution of the pathological targeting of vacuolation in the brains of clinically positive  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice (Fig 6.5). In contrast, no evidence of spongiform pathology, PrP<sup>d</sup> accumulation or reactive astrocytes was detected in the brains of surviving  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice (Figs 6.3 & 6.4).

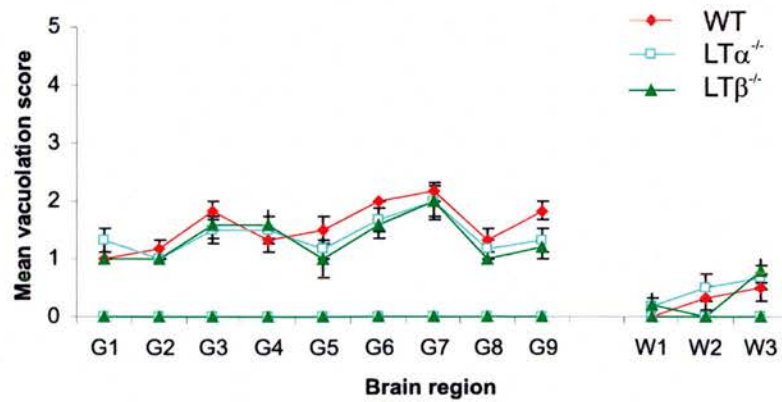
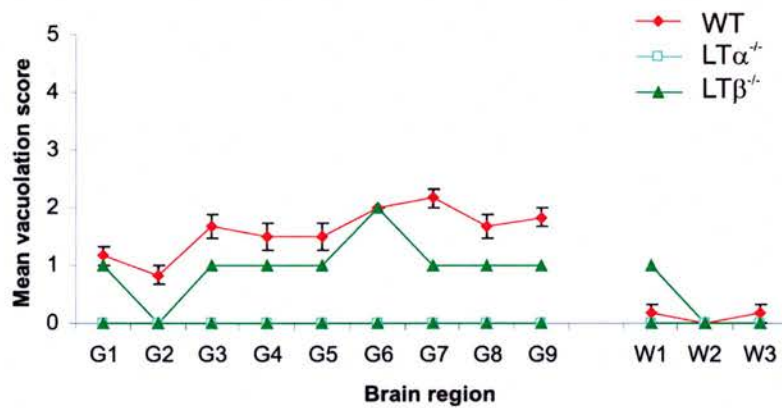
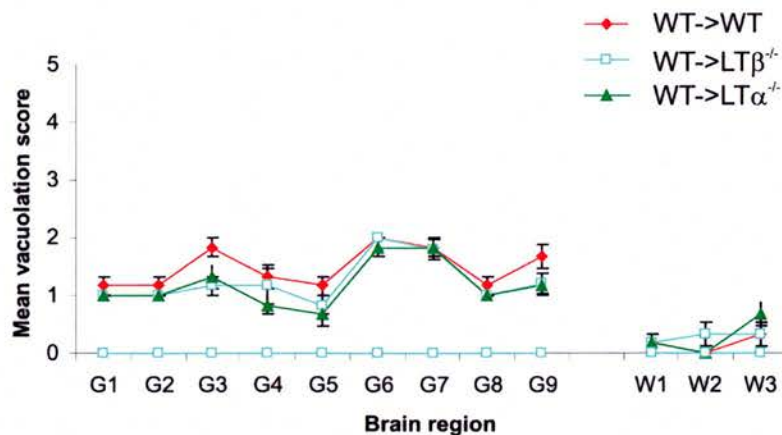
#### **6.3.4 Effect of LT deficiency on the early and late accumulation of PrP<sup>Sc</sup> in the spleen**

Within 70 days of i.p. inoculation of WT mice with the ME7 scrapie agent high levels of the proteinase K (PK) resistant, disease specific isomer of PrP (PrP<sup>Sc</sup>) are detected in spleen (Fig 6.6). In contrast, no PrP<sup>Sc</sup> was detected in spleens from  $LT\alpha^{-/-}$  mice or  $LT\beta^{-/-}$  mice at this time point. At the terminal stage of disease high levels of PrP<sup>Sc</sup> were detected in spleens from WT mice, but no PrP<sup>Sc</sup> was detected in spleens from either clinically positive or survivor  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice (Fig 6.7). Similar results were described in experiments involving  $TNF\alpha^{-/-}$  mice, which, like  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice, lack FDCs (Mabbott *et al.*, 2000b). This suggests that when FDCs are absent the ME7 scrapie agent is unable to accumulate in the spleen.

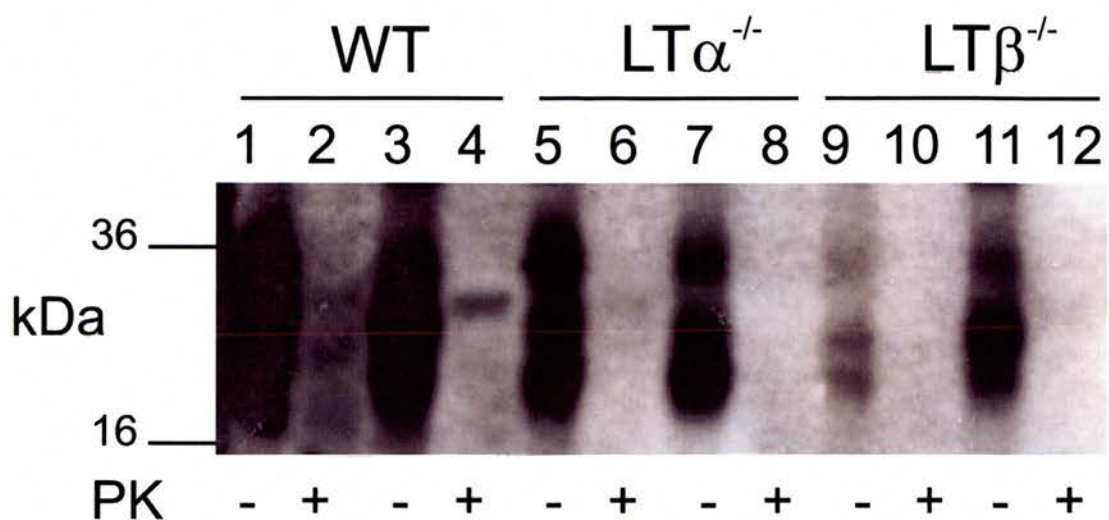
#### **6.3.5 Effect of CMLN deficiency on scrapie pathogenesis via i.p. inoculation**

WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were  $\gamma$ -irradiated and reconstituted with WT bone marrow to restore FDCs to the remaining lymphoid tissues (termed WT->WT mice, WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice, respectively). 35 days after bone marrow reconstitution mice were inoculated with 1.0 % dilution of ME7 scrapie



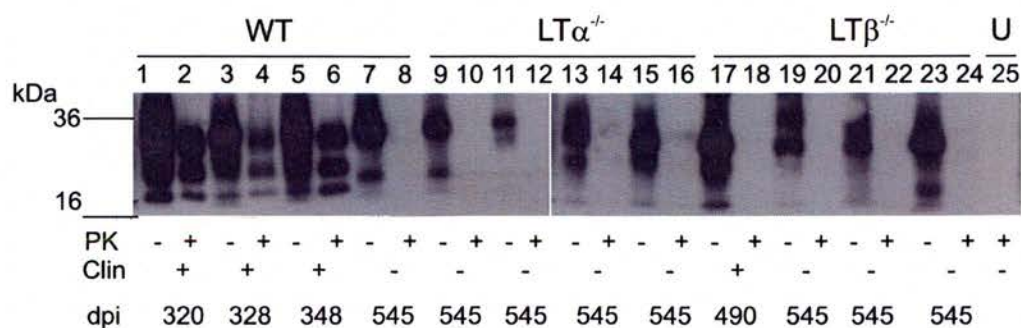
**a****b****c**

**Figure 6.5- Pathological targeting of vacuolation in the brains of mice terminally affected by ME7 scrapie or survivor mice.** Mice were inoculated intra-peritoneally with **a)** 0.1 % dilution of ME7 scrapie brain homogenate, **b)** 0.001 % dilution of ME7 scrapie brain homogenate or **c)** 0.1 % dilution of ME7 scrapie brain homogenate. Vacuolation in the brain was scored on a scale of 0-5 in the following grey matter (G1-G9) or white matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of the superior peduncles; W3, cerebral peduncles. Each point represents mean vacuolation score  $\pm$  S.E.M. for groups of 6-9 mice. WT, wild-type mice; LT $\alpha^{-/-}$ , lymphotoxin –  $\alpha$  deficient mice; LT $\beta^{-/-}$ , lymphotoxin –  $\beta$  deficient mice. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are WT mice, LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice respectively reconstituted with WT bone marrow.

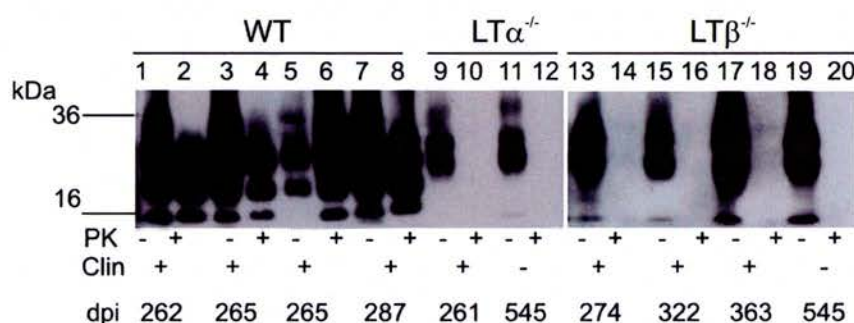


**Figure 6.6- PrP<sup>Sc</sup> accumulation in spleens of wild-type (WT) mice, lymphotoxin  $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice and lymphotoxin  $\beta$  deficient (LT $\beta^{-/-}$ ) mice 70 days after intra-peritoneal inoculation with ME7 scrapie.** Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in some spleens from WT mice. No PrP<sup>Sc</sup> was detected in spleens from either LT $\alpha^{-/-}$  mice or LT $\beta^{-/-}$  mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis.

**a**



**b**



**Figure 6.7- PrP<sup>Sc</sup> accumulation in spleens.** Spleens were collected from clinically affected wild-type (WT) mice, lymphotoxin  $\alpha$  deficient (LT $\alpha^{-/-}$ ) mice and lymphotoxin  $\beta$  deficient (LT $\beta^{-/-}$ ) mice, and survivor LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice after intra-peritoneal inoculation with **a**) 0.01 % dilution of ME7 scrapie brain homogenate or **b**) 1.0 % dilution of ME7 scrapie brain homogenate. Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in spleens from clinically affected WT mice, LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice. No PrP<sup>Sc</sup> was detected in spleens from survivor LT $\alpha^{-/-}$  mice or LT $\beta^{-/-}$  mice or from mice which had not been inoculated with scrapie (U). Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score either positive (+) or negative (-); dpi, days post inoculation on which tissues were taken for analysis



brain homogenate via i.p. injection. WT->WT mice developed disease with a mean incubation period of  $286 \pm 4$  days (Table 6.3) which was not significantly different from WT mice ( $p \leq 0.863$ ). Following the restoration of FDCs in remaining lymphoid tissues of WT->LT $\beta^{-/-}$  mice, all mice developed scrapie disease with a mean incubation period of  $290 \pm 11$  days (Table 6.3) similar to that of WT->WT mice ( $p \leq 0.957$ ). In contrast, eight out of 9 WT->LT $\alpha^{-/-}$  mice developed scrapie disease with one animal surviving to the time the experiment was terminated at 545 dpi. The mean incubation period for WT->LT $\alpha^{-/-}$  mice was  $337 \pm 6$ , which is significantly longer than the WT->WT mice and WT->LT $\beta^{-/-}$  mice ( $p \leq 0.011$ ). Histopathological analysis of the brain revealed characteristic spongiform pathology, PrP<sup>d</sup> accumulation and reactive astrocytes in the brains of all WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice which were scored clinically positive (Fig 6.8). The severity and distribution of the pathological targeting of the vacuolation in the brains of WT->WT mice was typical of ME7 scrapie. No significant differences were observed in the severity and distribution of the pathological targeting of vacuolation in the brains of clinically positive WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice (Fig 6.5). In contrast, no evidence of spongiform pathology, PrP<sup>d</sup> accumulation or reactive astrocytes was detected in the brain of the surviving WT->LT $\alpha^{-/-}$  mouse (Fig 6.8).

#### **6.3.6 Effect of CMLN deficiency on the early and late accumulation of PrP<sup>Sc</sup> in the spleen**

Within 70 days of inoculation with the ME7 scrapie agent, high levels of PrP<sup>Sc</sup> were detected in the spleens of WT->WT mice (Fig 6.9). PrP<sup>Sc</sup> was detected only in some spleens from WT->LT $\alpha^{-/-}$  mice and in none of the WT->LT $\beta^{-/-}$  mice at this time

point. At the terminal stage of disease high levels of PrP<sup>Sc</sup> were detected in spleens from all clinically positive WT->WT mice, WT->LT $\alpha$ <sup>-/-</sup> mice and WT->LT $\beta$ <sup>-/-</sup> mice and PrP<sup>Sc</sup> was also detected in the spleen of the survivor WT->LT $\alpha$ <sup>-/-</sup> mouse (Fig 6.10).

	Presence at inoculation			Incidence <sup>1</sup>	Mean incubation period
	SPL	CMLNs	FDCs		
WT->WT	+	+	+	9/9	286 ± 4
WT->LTα <sup>-/-</sup>	+	-	+	8/9	337 ± 16, 1 ≤ 503
WT->LTβ <sup>-/-</sup>	+	-	+	7/7	290 ± 11*

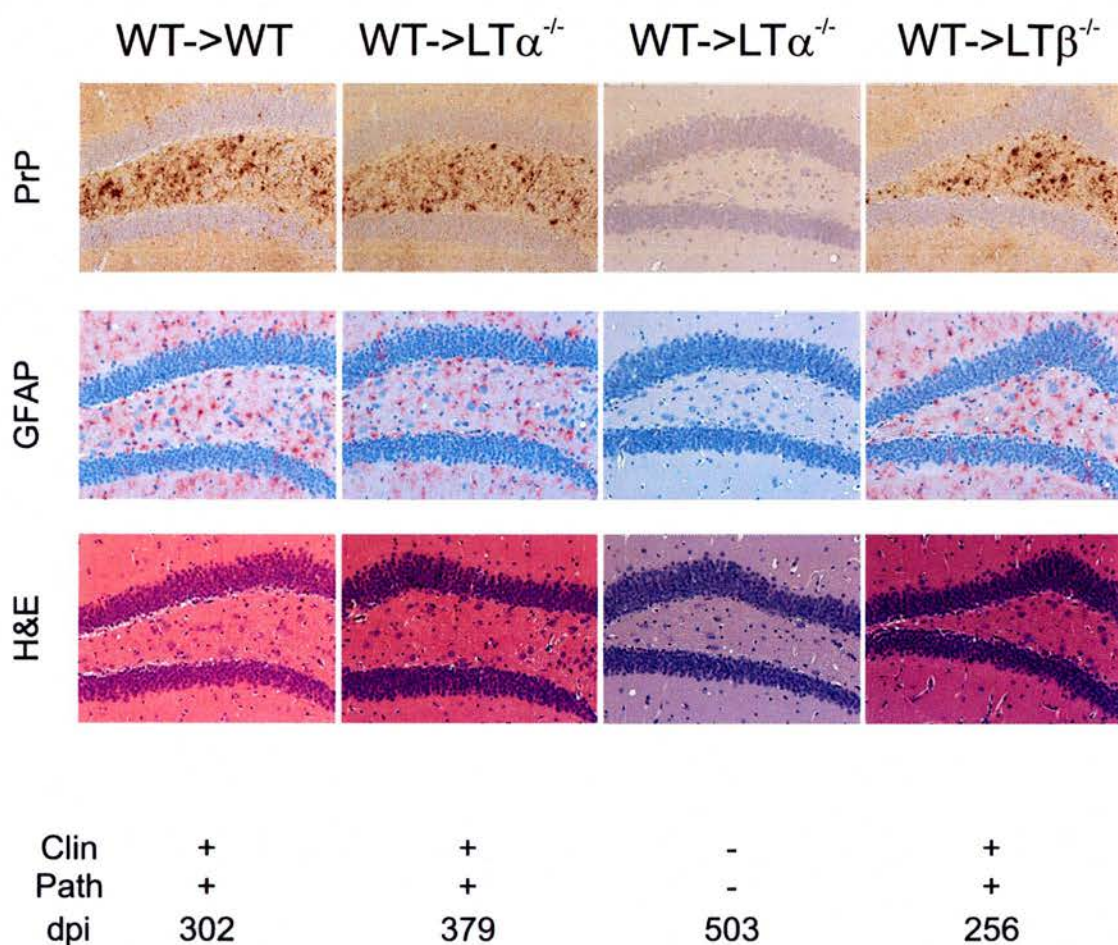
**Table 6.3 – Effect of CMLN deficiency on scrapie susceptibility after inoculation via intra-peritoneal injection.**

1. Incidence = number of animals affected/ number of animals tested.

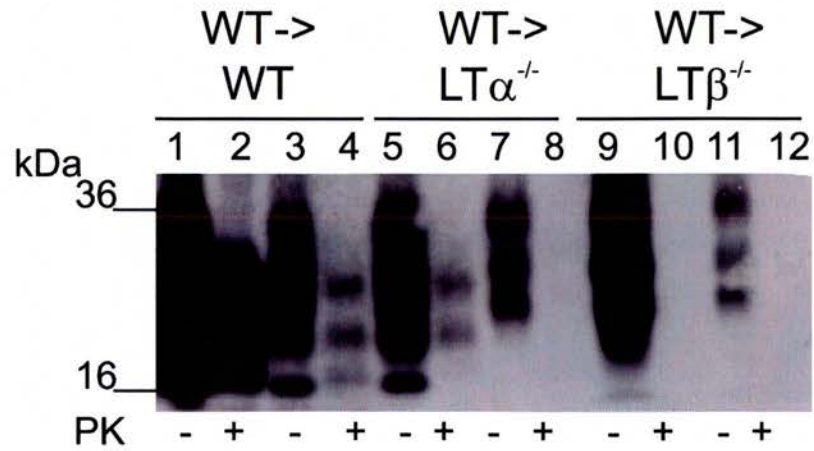
SPL = Spleen; CMLNs = cranial mediastinal lymph nodes; FDCs = follicular dendritic cells; WT->WT, WT->LTα<sup>-/-</sup> and WT->LTβ<sup>-/-</sup> are wild-type mice, lymphotoxin -α deficient mice and lymphotoxin -β deficient mice respectively, reconstituted with wild-type bone marrow. + = presence, - = absence

\*  $p \geq 0.05$  when compared to the mean incubation period of control mice indicating no significant difference

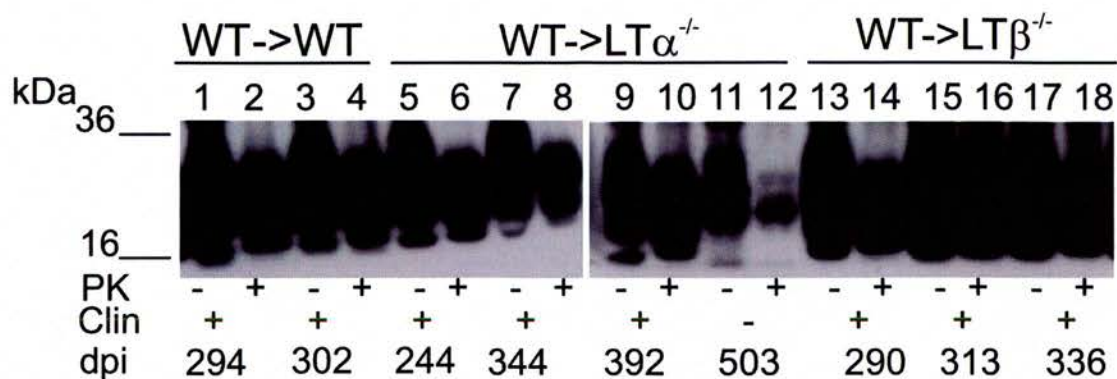




**Figure 6.8- Histological analysis of brain tissue from terminally scrapie affected mice or survivor mice inoculated intra-peritoneally with 1.0 % dilution of ME7 scrapie brain homogenate.** Large disease specific prion protein (PrP<sup>d</sup>) accumulations were detected in the hippocampi of all mice which developed clinical signs of scrapie (brown; upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red; middle row) showed diffuse gliosis in the hippocampi of all terminally scrapie affected mice. Adjacent sections were also stained with haematoxylin and eosin (H & E; lower row) and extensive vacuolation was displayed in all mice terminally affected by scrapie. None of these pathological signs of scrapie disease were detected in the brains of clinically negative mice at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification X200. Clin, clinical score, either positive (+) or negative (-); Path, pathological score, either positive (+) or negative (-); dpi, days post-inoculation on which tissues were taken for analysis. WT->WT , WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are wild-type mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice respectively, reconstituted with wild-type bone marrow.



**Figure 6.9- PrP<sup>Sc</sup> accumulation in spleens of WT->WT mice, WT->LT $\alpha^{-/-}$  mice or WT->LT $\beta^{-/-}$  mice 70 days after intra-peritoneal inoculation with ME7 scrapie.** Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in spleens from WT->WT mice and some WT->LT $\alpha^{-/-}$  mice. No PrP<sup>Sc</sup> was detected in spleens from WT->LT $\beta^{-/-}$  mice. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are wild-type mice, lymphotoxin  $\alpha$  deficient mice and lymphotoxin  $\beta$  deficient mice respectively, reconstituted with wild-type bone marrow.



**Figure 6.10- PrP<sup>Sc</sup> accumulation in spleens of clinically affected WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice or the surviving WT->LT $\alpha^{-/-}$  mouse after intra-peritoneal inoculation with ME7 scrapie.** Proteinase K (PK) resistant, disease specific prion protein (PrP<sup>Sc</sup>) was detected in spleens of clinically affected WT->WT mice, WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice. PrP<sup>Sc</sup> was also detected in the spleen of the surviving WT->LT $\alpha^{-/-}$  mouse. Samples were treated in the presence (+) or absence (-) of PK prior to electrophoresis. Clin, clinical score, either positive (+) or negative (-); dpi, days post inoculation on which the tissues were taken for analysis. WT->WT, WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  are wild-type, lymphotoxin  $\alpha$ -deficient mice and lymphotoxin  $\beta$ -deficient mice respectively, reconstituted with wild-type bone marrow.



## **6.4 Discussion**

Inoculation via i.p. injection is a commonly used route for the investigation of the peripheral pathogenesis of TSEs. The peritoneal cavity is thought to be drained by the CMLNs.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack the CMLNs and lack FDCs in remaining lymphoid tissues. These mice and WT control mice were inoculated with the ME7 strain of the scrapie agent via i.p. injection.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice exhibited reduced susceptibility and delayed onset of disease compared to WT mice, demonstrating that neuroinvasion is less efficient in the absence of both the local lymph nodes and FDCs in remaining lymphoid tissues.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice were also reconstituted with WT bone marrow to restore FDCs to the remaining lymphoid tissues and challenged with scrapie to determine the potential involvement of the lymph nodes draining the peritoneal cavity in scrapie pathogenesis via the i.p. route. WT-> $LT\alpha^{-/-}$  mice displayed reduced susceptibility and delayed onset of disease whereas WT-> $LT\beta^{-/-}$  mice were fully susceptible to scrapie and developed disease in the same time frame as WT->WT control mice. This demonstrated that the CMLNs were not important in scrapie pathogenesis and raised the possibility that other lymphoid tissues, present in WT-> $LT\beta^{-/-}$  mice but absent from WT-> $LT\alpha^{-/-}$  mice were playing an important role.

Within 10-15 min of injection of Indian ink into the peritoneal cavity, the lymphatic capillaries on the peritoneal surface of the diaphragm stain strongly blue-black (Marco *et al.*, 1992; Shinohara, 1997). The diaphragm is thought to act as a “lymphatic sieve” whereby fluid and small particles from the peritoneal cavity are taken up non-specifically by stomata in lymphatic capillaries on the peritoneal

surface of the diaphragm. These stomata are peculiar to the peritoneal surface as Indian ink placed on the peritoneal surface will be quickly taken up, in contrast only small amounts of Indian ink are taken up slowly when placed on the pleural surface of the diaphragm (Shinohara, 1997). The lymphatic capillaries of the diaphragm converge into two lymphatic vessels which enter the thoracic cavity at the level of the oesophageal hiatus (the opening in the diaphragm through which passes the oesophagus and the two vagus nerves; Marco *et al.*, 1992). Lymph is thought to flow from the diaphragm to the thoracic duct via the lymph nodes of the thoracic cavity. The accumulation of Indian ink or *Listeria monocytogenes* has been demonstrated at early time points in the CMLNs prior to detection in the spleen and other lymph nodes after i.p. injection (Hill, 1976; Marco *et al.*, 1992). This demonstrates that CMLNs drain the peritoneal cavity and would be the first lymphoid tissues to receive the scrapie agent after i.p. inoculation.

As discussed in section 3.4 the development of most secondary lymphoid tissue is critically dependent upon  $LT\alpha_1\beta_2$  signalling through  $LT\beta$  receptor ( $LT\beta R$ ) during gestation. As a consequence, CMLNs do not form in mice where this signalling has been interrupted during gestation by genetic deficiency of  $LT\alpha$  (Rennert *et al.*, 1997) or  $LT\beta$  (data presented here). As discussed in section 4.4 the remaining lymphoid tissues of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack FDCs and display disorganised microarchitecture.  $\gamma$ -irradiation and WT bone marrow reconstitution of these mice results in the restoration of FDC networks and organisation of lymphoid tissue microarchitecture without inducing the formation of the missing lymphoid tissues (section 4.4).

Several groups have utilised  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice to determine the importance of FDCs in TSE pathogenesis after i.p. inoculation. The results of these studies have not been consistent. One study found there to be no difference between  $LT\beta^{-/-}$  mice and immunocompetent control mice when inoculated with a mouse-passaged strain of vCJD (Manuelidis *et al.*, 2000). Other studies, however, found  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice to have reduced susceptibility or to be resistant to the RML strain of scrapie agent (Oldstone *et al.*, 2002; Prinz *et al.*, 2002). These differences could be explained by the different strains of TSE agent and different doses of inocula used. In light of this conflicting evidence the work described in this thesis determined the pathogenesis of the ME7 strain of scrapie agent in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice displayed reduced susceptibility to the ME7 scrapie agent. However, some cases did occur in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice demonstrating the presence of an FDC-independent route of neuroinvasion, perhaps involving direct uptake of the scrapie agent by nerve endings in the peritoneal cavity. This is consistent with previous reports of cases of clinical scrapie in FDC-deficient  $TNF\alpha^{-/-}$  mice after i.p. inoculation with ME7 scrapie agent (Mabbott *et al.*, 2000b). However, not all  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice or  $TNF\alpha^{-/-}$  mice inoculated contracted disease and the incubation periods of disease in those that did varied considerably from 216 – 503 days, in contrast to the small range in WT mice (typically 262 – 321 days). Clearly the FDC-independent route of neuroinvasion occurring in  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice and  $TNF\alpha^{-/-}$  mice is much less efficient than the FDC-dependent route in WT mice.



In the studies utilising  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice mentioned above (Manuelidis *et al.*, 2000; Oldstone *et al.*, 2002; Prinz *et al.*, 2002) the separate roles that lymphoid tissues and FDCs play in TSE pathogenesis were not and cannot be distinguished from each other. In this chapter, by reconstituting  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow and thus restoring terminal FDC differentiation, data presented here shows the effect that a specific lack of lymphoid tissue has on pathogenesis of ME7 scrapie after i.p. inoculation. WT-> $LT\beta^{-/-}$  mice all contracted disease with no significant difference in the time of onset of disease when compared to WT->WT mice. As WT-> $LT\beta^{-/-}$  mice lack CMLNs this demonstrates that the CMLNs are not important in scrapie pathogenesis after i.p. inoculation and that neuroinvasion can occur from another site. Intriguingly, there was delayed onset and decreased susceptibility in WT-> $LT\alpha^{-/-}$  mice despite the presence of mature FDCs in remaining lymphoid tissues.

Reconstitution adult  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow should restore the functions of both  $LT\alpha_1\beta_2$  and  $LT\alpha_3$  equally in WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice as these cytokines are expressed by bone marrow derived cells (Gommerman & Browning, 2003). This implies that the only difference between WT-> $LT\alpha^{-/-}$  mice and WT-> $LT\beta^{-/-}$  mice is the number of remaining lymphoid tissues. This raises the possibility that, although the CMLNs were clearly absent from both types of mice, there may be another lymph node draining the peritoneum which was present in WT-> $LT\beta^{-/-}$  mice and absent in WT-> $LT\alpha^{-/-}$  mice, perhaps the other lymph nodes within the thoracic cavity such as the caudal mediastinal lymph nodes or tracheobronchial lymph nodes (Broeck *et al.*, 2006). Thus the difference in

disease outcome between the two types of mice might be explained by the restoration of FDCs (and therefore efficient neuroinvasion) to other lymph nodes in WT->LT $\beta^{-/-}$  mice which are not present in WT->LT $\alpha^{-/-}$  mice. However, to date, the presence or absence of caudal mediastinal lymph nodes or tracheobronchal lymph nodes has not been confirmed in either LT $\alpha^{-/-}$  mice or LT $\beta^{-/-}$  mice, nor is there any data on the involvement of these lymph nodes in the lymphatic drainage of the peritoneum. It has been reported that LT $\beta^{-/-}$  mice have a single lymph node in the sacral region, in a different anatomical location to the conventional sacral lymph nodes found in WT mice (Soderberg *et al.*, 2004). Therefore, it is also plausible that a similar modification could have occurred in the thoracic region of LT $\beta^{-/-}$  mice used in the experiments in this thesis, although this was not noticed on examination of the thoracic region of these mice after the injection of Indian ink.

The stromal cells of LT $\alpha^{-/-}$  mice will not express LT $\alpha$ , however, the stromal cells of the LT $\beta^{-/-}$  mice will. Theoretically, it is also possible that a cell type that relies on LT $\alpha$  expression by a stromal cell could be causing the differences seen when WT->LT $\alpha^{-/-}$  mice and WT->LT $\beta^{-/-}$  mice were challenged with scrapie via the i.p. route. However, there is no evidence that such a cell exists as LT $\alpha$  expression appears to be restricted to bone marrow-derived cells (Gommerman & Browning, 2003).

Data here clearly identify an FDC-independent route of neuroinvasion after i.p. inoculation but this route is clearly much less efficient than the FDC-dependent route. It is plausible that neuroinvasion normally occurs by both FDC-independent and FDC-dependent methods. Likewise, the crucial function of FDCs in scrapie

pathogenesis may be to amplify the scrapie agent within the lymphoid tissues above a threshold such that neuroinvasion can efficiently occur. WT->LT $\beta^{-/-}$  mice have more remaining lymphoid tissues than WT->LT $\alpha^{-/-}$  mice and, therefore, a higher total number of FDCs. This difference might contribute to the decreased susceptibility and increased incubation periods observed in WT->LT $\alpha^{-/-}$  when compared to WT->WT mice and WT->LT $\beta^{-/-}$  mice.

After inoculation via i.p. injection with the ME7 scrapie agent, neuroinvasion does not occur solely from the spleen as splenectomy prolongs the onset, but does not prevent disease from occurring (Fraser & Dickinson, 1978). Similarly, results presented here show that neuroinvasion is delayed or even prevented in mice which have a spleen containing FDCs (WT->LT $\alpha^{-/-}$  mice) but lack other lymphoid tissues. Taken together these results show that although neuroinvasion does occur from the spleen after i.p. inoculation this is not the only location neuroinvasion occurs from and other tissues also play an important role in supporting neuroinvasion.



# 7

## General discussion

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## **7. General Discussion**

### **7.1. The role of draining lymphoid tissues in TSE agent neuroinvasion**

Previous studies have demonstrated that TSE infectivity and PrP<sup>Sc</sup> accumulate in the lymphoid tissues draining the site of inoculation prior to their detection in non-draining lymphoid tissues and the CNS in a number of natural (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2000; Keulen *et al.*, 1999) and experimental (Mabbott *et al.*, 2003; Mohan *et al.*, 2005a) studies. The main aim of this thesis was to determine the importance of this initial accumulation phase in the draining lymphoid tissues in TSE agent neuroinvasion. The approach taken here was to utilise mice which lack various lymphoid tissues, such as LT $\alpha$ <sup>-/-</sup> mice which possess only the spleen and nasal associated lymphoid tissue (NALT; Banks *et al.*, 1995; Fukuyama *et al.*, 2002; Togni *et al.*, 1994), LT $\beta$ <sup>-/-</sup> mice which, as well as containing spleen and NALT, also have mesenteric lymph nodes (MLNs) and cervical lymph nodes (CLNs; Fukuyama *et al.*, 2002; Koni *et al.*, 1997) and the progeny of mice treated *in utero* with LT $\beta$ R-Ig on day 11.5 of gestation which contain spleen, NALT, MLNs, CLNs and also the lumbar and sacral lymph nodes (Rennert *et al.*, 1997; Rennert *et al.*, 1996). These mice were inoculated with the scrapie agent via different routes of inoculation and the effect on TSE disease progression analysed.

Mice which have no lymphoid tissues in the intestine were resistant to scrapie challenge after oral inoculation (Chapter 4). After inoculation via scarification of the skin of the thigh mice which lacked the draining inguinal lymph node were less susceptible to scrapie challenge than immunocompetent controls (Chapter 5). After

intra-peritoneal inoculation Lymphotoxin (LT) –  $\alpha$  deficient mice (LT $\alpha^{-/-}$  mice) which had been reconstituted with wild-type (WT) bone marrow (WT->LT $\alpha^{-/-}$  mice) were less susceptible and had delayed onset of disease compared to WT mice. LT $\beta^{-/-}$  mice reconstituted with WT bone marrow (WT->LT $\beta^{-/-}$ ) mice were as susceptible and developed disease in the same timeframe as WT mice. This finding raised the possibility that lymphoid tissue present in WT->LT $\beta^{-/-}$  mice and absent from WT->LT $\alpha^{-/-}$  mice (or, theoretically, a cell type relying on LT $\alpha$  expression by a stromal derived cell) was important in supporting neuroinvasion (Chapter 6). This demonstrates that the accumulation of the TSE agent in the draining lymphoid tissues is important, to a greater or lesser extent depending on the route of inoculation.

The initial spread of the TSE agent from the site of inoculation to the draining lymphoid tissues most likely occurs via the lymphatic vasculature. If this occurred by blood borne transport it would be expected that infectivity and PrP<sup>Sc</sup> accumulation would be detected in all lymphoid tissues more or less simultaneously. Therefore, results showing accumulation in draining lymphoid tissues prior to detection in non-draining lymphoid tissues (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2000; Keulen *et al.*, 1999; Mabbott *et al.*, 2003; Mohan *et al.*, 2005a) demonstrate that the initial spread of the TSE agent occurs most likely via the lymphatic system rather than haematogenously.

Once the TSE agent has entered the body a competitive state exists whereby phagocytic cells destroy the agent while cells in the lymphoid tissues replicate it. Normally, when draining lymphoid tissues are present replication prevails resulting



in neuroinvasion and the subsequent development of TSE disease. However, results presented in this thesis show that when the draining lymphoid tissues are missing, the prevailing situation is altered to a greater or lesser extent, depending on the route of inoculation.

After oral inoculation it is likely that very little of the inoculum crosses the intestinal epithelial barrier. Some of the inoculum may be digested by enzymes in the stomach or small intestine (Jeffrey *et al.*, 2006; Mishra *et al.*, 2004) and most of the inoculum that reaches the intestine will be passed from the body. Thus in a normal situation the very little of the starting inoculum will reach the Peyer's patches (PPs). Although there is no direct *in vivo* evidence, it has been postulated that the TSE agent may cross the intestinal epithelium through uptake by M cells (Heppner *et al.*, 2001), dendritic cells (Huang *et al.*, 2002) or enterocytes (Mishra *et al.*, 2004; Morel *et al.*, 2005). WT bone marrow reconstituted  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice should be equally as capable as WT mice of taking up the scrapie agent as they have M cells, dendritic cells and intact lymphatics (Chapter 3; Kabashima *et al.*, 2005; Chapter 3; Tumanov *et al.*, 2004; Wu *et al.*, 1999). However, these mice do not develop disease when inoculated orally (chapter 4). After oral inoculation, it is likely that destruction of the agent by phagocytes prevails allowing complete clearance of the agent and preventing subsequent disease. Thus it is plausible that in the absence of the PP the small amount of inoculum that makes it across the intestinal epithelium is destroyed before it reaches any other lymphoid tissues (such as the MLNs in WT- $\rightarrow$  $LT\beta^{-/-}$  mice).

With both the scarification and i.p. routes it may be assumed that almost the entire inoculum will enter the lymphatic vasculature as inoculation via these routes does not involve passage through the stomach or intestine. Thus the difference in whether destruction or replication of the agent prevails in the absence of the draining lymphoid tissues between the oral compared to the scarification or i.p. routes may be attributed to the amount of TSE infectivity which reaches the draining lymphatic vessels.

The importance of follicular dendritic cells (FDCs) in TSE pathogenesis has been demonstrated by experiments which have shown that a temporary (Mabbott *et al.*, 2000a; Mabbott *et al.*, 2002; Mabbott *et al.*, 2003; Montrasio *et al.*, 2000) or permanent (Mabbott *et al.*, 2000b; Prinz *et al.*, 2002) absence of FDCs at the time of inoculation delays or prevents the onset of disease. As both  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack FDCs, prior to use in scrapie inoculation experiments, these mice were reconstituted with WT bone marrow. This restored terminal FDC differentiation without inducing the formation of the missing lymphoid tissues. Thus results obtained using these mice could be attributed to the lack of lymphoid tissues, rather than a dual lack of both lymphoid tissues and FDCs. Studies in this thesis and elsewhere have demonstrated an FDC independent route of neuroinvasion. Inefficient neuroinvasion was able to occur after i.p. inoculation of mice which lacked FDCs through genetic deficiency of  $LT\alpha$ ,  $LT\beta$  (Chapter 6),  $TNF\alpha$  (Mabbott *et al.*, 2000b) or  $TNFR1$  (Klein *et al.*, 1997). The reason for this could be the direct uptake of the scrapie agent by nerve endings in the peritoneal cavity or skin bypassing the need for replication of the agent in the periphery. It has also been

suggested that as macrophages in  $\text{TNF}\alpha^{-/-}$  or  $\text{TNFR1}^{-/-}$  mice cannot be properly activated, the lack of FDCs is perhaps compensated for by replication of the scrapie agent in dormant macrophages (Prinz *et al.*, 2002). This route of neuroinvasion is much less efficient than the FDC-dependent route.

The work presented in this thesis has demonstrated that lymphoid tissue within the intestine is crucial for neuroinvasion to occur. However, as discussed in section 4.4 it is not known whether the lack of lymphoid tissue in the intestine directly prevents neuroinvasion from occurring, or if the scrapie agent is simply prevented from spreading to other sites from which neuroinvasion occurs. Mice genetically deficient for tumour necrosis factor-related activation induced cytokine (TRANCE) develop PPs and spleens but do not develop any other lymphoid tissue (Dougall *et al.*, 1999; Kong *et al.*, 1999). Utilising these mice in scrapie pathogenesis experiments would, therefore, be a way of addressing this question, particularly if splenectomy was carried out prior to inoculation. Thus, if neuroinvasion occurs directly from PPs within the intestine after oral infection, splenectomised  $\text{TRANCE}^{-/-}$  mice should be as susceptible, and develop disease in the same time frame, as WT mice. If neuroinvasion occurs from a combination of lymphoid tissues, such as PPs and spleen, after oral inoculation then splenectomised  $\text{TRANCE}^{-/-}$  mice would take longer to develop disease, or have a lower incidence compared to WT mice. In this case, non-splenectomised  $\text{TRANCE}^{-/-}$  mice would be as susceptible and develop disease in the same time frame as WT mice. If neuroinvasion occurs from the spleen alone after oral inoculation then splenectomised  $\text{TRANCE}^{-/-}$  mice or WT mice would be resistant to disease, however this is unlikely as various pieces of evidence would



suggest that neuroinvasion does, at least to some extent, occur from the intestine itself (Beekes & McBride, 2000; Kimberlin & Walker, 1989; Mabbott *et al.*, 2003). Performing the experiments described above would resolve this issue.

Another approach to this experiment could be to inoculate WT->LT $\alpha^{-/-}$  mice with the scrapie agent at twelve weeks post bone marrow reconstitution. LT $\alpha^{-/-}$  mice lack all gut associated lymphoid tissues, but at this time point post reconstitution mature ILFs have developed (Chapter 3; Lorenz *et al.*, 2003; McDonald *et al.*, 2005). Thus a model could be created where lymphoid tissue is present in the intestine (mILFs) and spleen but at no other sites in the body. These mice could then be utilised as described above for TRANCE $^{-/-}$  mice to determine whether neuroinvasion occurs directly from the intestine. It has previously been reported that some LT $\alpha^{-/-}$  mice do possess reduced numbers of MLNs (Fu *et al.*, 1997). Thus TRANCE $^{-/-}$  mice represent a cleaner system to address this question. However, oral scrapie agent inoculation of both WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  mice 12 weeks post bone marrow reconstitution would be interesting to demonstrate that the mILFs of WT->LT $\alpha^{-/-}$  and WT->LT $\beta^{-/-}$  mice were capable of supporting neuroinvasion in the same way described for *in utero* LT $\beta$ R-Ig treated mice (PP-deficient mice) in chapter 4 of this thesis.

One could also argue that the results detailed in this thesis are not due to the specific lack of lymphoid tissues draining the site of inoculation but rather to the lower total number of FDCs present in the mice. Thus, SCID mice which lack all FDCs are resistant while wild-type mice with maximum numbers of FDCs are fully susceptible

(Brown *et al.*, 1997c; Fraser *et al.*, 1996; O'Rourke *et al.*, 1994; Taylor *et al.*, 1996b). Mice which lack some lymphoid tissues and have, therefore, an intermediate number of FDCs vary in their response to infection as demonstrated by the various resistance, decrease in susceptibility or increase incubation period outcomes described here. If it were true that these results were due to a decrease in the overall number of FDCs rather than a lack of lymphoid tissues (and, therefore, FDCs) at specific anatomical locations, this would imply that neuroinvasion crucially occurs from all lymphoid tissues regardless of the route of inoculation. Therefore a lack of lymphoid tissues anywhere in the body would have an impact on disease via any route of inoculation. This is not true, however, as the induction of mILF formation in the intestines of *in utero* LT $\beta$ R-Ig treated mice rendered the mice fully susceptible to oral scrapie agent challenge (Chapter 4) despite the absence of many of the non-draining lymphoid tissues and, therefore, a decreased total number of FDCs compared to control mice. Thus, it is reasonable to conclude that the results described in this thesis can be attributed to the absence of the lymphoid tissues draining the site of inoculation.

This is not to say that accumulation and replication of the agent and neuroinvasion does not occur from non-draining lymphoid tissue. Indeed, results presented here would suggest that following some routes of inoculation neuroinvasion from non-draining lymphoid tissues can compensate for the absence of the draining lymphoid tissues, but this route of neuroinvasion is much less effective (Chapters 5 & 6). The accumulation and replication of the agent in draining lymphoid tissues is, therefore, more important than in non-draining lymphoid tissues.

## **7.2 Why are draining lymphoid tissues important in TSE pathogenesis?**

Data presented in this thesis show that the draining lymphoid tissue is important for efficient neuroinvasion. Evidence suggests that the FDCs are the crucial component within the lymphoid tissues for the accumulation and replication of the scrapie agent. PrP<sup>d</sup> specific immunostaining in lymphoid tissues co-localises with FDC specific immunostaining in both natural (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2000; Hilton *et al.*, 1998) and experimental (Brown *et al.*, 1999b; Mabbott *et al.*, 2000b; McBride *et al.*, 1992; Sigurdson *et al.*, 1999) cases of disease. Furthermore, a temporary (Mabbott *et al.*, 2000a; Mabbott *et al.*, 2002; Mabbott *et al.*, 2003; Montrasio *et al.*, 2000) or permanent (Mabbott *et al.*, 2000b; Prinz *et al.*, 2002) absence of FDCs at the time of inoculation delays or prevents the onset of disease. Thus it is logical to conclude that when lymphoid tissues draining the site of inoculation are missing it is the lack of FDCs local to the site of inoculation that impacts on the development of disease.

However, there may be other reasons for the lack of draining lymphoid tissues having an impact on the development of TSE disease. For example, although experiments have demonstrated that WT bone marrow reconstitution of LT $\alpha$ <sup>-/-</sup> mice and LT $\beta$ <sup>-/-</sup> mice restores the migration of dendritic cells into lymphoid tissues remaining in these mice (Wu *et al.*, 1999), it is not known what happens at sites where the lymphoid tissue itself is missing. The factors involved in the migration of dendritic cells into lymphatic vessels are not fully understood. CCR7 expression by dendritic cells is important in this process and a ligand for CCR7 (Martin-Fontecha *et*



*al.*, 2003) CCL21, is expressed by peripheral lymphatic vessels (Chen *et al.*, 2002) suggesting that an interaction between these two molecules may facilitate this process. If this is the case, dendritic cell migration into lymphatic vessels should not be affected by the absence of the local lymph nodes. However, there is currently no evidence that this interaction does take place, nor is there any data on the factors which regulate the expression of CCL21 by lymphatic endothelial cells. Thus it is possible that the absence of the local lymphoid tissue results in the impaired migration of cells carrying the scrapie agent from the site of inoculation to the lymphoid tissues. This would prevent the early accumulation and replication of the agent such that the agent could be destroyed before it reaches more distant remaining lymphoid tissues.

### **7.3 Spread of the TSE agent from the draining lymphoid tissues to the CNS**

The spread of TSE agents from the sites of replication in lymphoid tissue to the CNS is thought to occur via the peripheral nervous system. Early investigations in mice found that after peripheral challenge replication of scrapie in the CNS consistently began in the thoracic region of the spinal cord with replication in the brain and other parts of the spinal cord occurring significantly later (Kimberlin & Walker, 1980). This suggested that neuronal rather than haematological spread was occurring as this ordered sequence of events would not be expected if the agent was spreading via the bloodstream.

Further evidence for neural spread came from experiments showing that the expression of PrP<sup>C</sup> in the peripheral nerves is required for the development of disease (Blattler *et al.*, 1997; Glatzel & Aguzzi, 2000). When the scrapie agent was inoculated directly into peripheral nerves, mice over expressing PrP<sup>C</sup> were found to develop disease faster than control mice suggesting that peripheral nerves could transport the scrapie agent to the CNS and that this transport occurred in a PrP<sup>C</sup> dependent manner, although this could be due to the increased availability of PrP<sup>C</sup> for conversion to PrP<sup>Sc</sup> (Glatzel & Aguzzi, 2000). No disease was detected in the brains of PrP<sup>-/-</sup> mice reconstituted with PrP<sup>+/+</sup> neurones in the brain only when challenged peripherally with scrapie (Blattler *et al.*, 1997). PrP<sup>+/+</sup> lymphohaemopoietic stems cells were then given to allow potential replication in the lymphoid tissues and spread via the bloodstream. Once again resistance to scrapie was detected suggesting that the spread of scrapie to the CNS occurs via the peripheral nervous system, not via the blood stream, and was dependent on the nerves expressing PrP<sup>C</sup>. It is possible to argue that no disease developed in the latter study (Blattler *et al.*, 1997) because the FDCs, which are not of haemopoietic origin, would be PrP<sup>-/-</sup>, and therefore, would not support scrapie replication. However, it is worth noting that the strain of scrapie used in this study was RML, which in some circumstances accumulate in lymphoid tissues in the absence of PrP expression by FDCs. (Klein *et al.*, 1997). A further demonstration of the involvement of peripheral nerves in TSE pathogenesis has been proposed through removal or over expression of them (Glatzel *et al.*, 2001). Removal of nerves before peripheral scrapie challenge was found to delay the onset of disease in the CNS, whereas hyperinnervation reduced the incubation period.

Effort has also been put into trying to determine specifically which nerves are involved in the spread of the TSE agent from the periphery to the CNS. Studies of the enteric nervous system have revealed that both the sympathetic and the parasympathetic nervous systems may be involved as PrP<sup>d</sup> has been found in the efferent fibres of these nerves prior to detection in the CNS (Baldauf *et al.*, 1997; Beekes *et al.*, 1996; Beekes & McBride, 2000; Beekes *et al.*, 1998; McBride & Beekes, 1999; McBride *et al.*, 2001). This is further supported by work which involved sympathectomies (either permanent or temporary) which delayed or in some cases even prevented the onset scrapie symptoms (Glatzel *et al.*, 2001). The involvement of the enteric nervous system in natural TSE disease has been shown by the accumulation of PrP<sup>d</sup> in these nerves in cases sheep scrapie (Heggebo *et al.*, 2003), humans with vCJD (Haik *et al.*, 2003) and deer affected by CWD (Sigurdson *et al.*, 2001).

It is not clear how the TSE agent reaches the peripheral nerves from the FDCs within the lymphoid tissue. In the spleen FDCs reside in the periphery of the white pulp and nerves are closely associated with the blood vessels, thus direct transfer between the two is unlikely (Defaweux *et al.*, 2005). Sympathetic innervation of lymph nodes has been observed but these innervate the cortical and medullary regions of the lymph nodes and nerve fibres have not been observed in germinal centres within lymph nodes (Mignini *et al.*, 2003). It is possible that another cell type may be responsible for the transfer of TSEs from FDCs to the peripheral nerves. Experiments showed that in RAG1<sup>-/-</sup> mice that lack B and T lymphocytes, and therefore, mature FDCs, injection of infected DCs was able to cause TSE disease



(Aucouturier *et al.*, 2001). There could be no involvement of lymphocytes or FDCs in this model since none were present in lymphoid tissue of RAG1<sup>-/-</sup> mice. Further work is required, however, before the conclusion that DCs carry TSE agents to the nervous system in the course of natural infections can be reached or that DCs are responsible for the infection of peripheral nerves when FDCs and lymphocytes are present. It is also possible that release of the TSE agent by exosomes from FDCs or other infected cells within lymphoid tissues may facilitate the infection of nerves of the lymphoid tissue as exosomes in infected cells have been found to release PrP<sup>Sc</sup> (Fevrier *et al.*, 2004).

Chemokines are important in directing FDCs to the correct microenvironment within the spleen. In mice deficient in the CXCR5 chemokine receptor (CXCR5<sup>-/-</sup> mice) this direction does not happen and FDCs aggregate around splenic nerves (Prinz *et al.*, 2003a). When these mice were challenged peripherally with scrapie, infectivity was found in the spinal cord 30 days earlier than in wild-type mice. This suggests the involvement in scrapie pathogenesis of both FDCs and splenic nerves, as, if either one was not necessary, putting them physically closer together would not affect the speed in which neuroinvasion occurs. Thus it would seem that the TSE agent does reach the peripheral nerves from FDCs, but the mechanism involved remains obscure. In PPs FDC networks are situated distal from the intestinal lumen and lie close to nerve fibres that run along the intestinal wall (Beekes & McBride, 2000). Likewise, in this thesis, FDCs in mILFs are shown to be similarly situated (Chapter 4). As the distance between FDCs and nerves appears to modulate the rate of neuroinvasion (Prinz *et al.*, 2003a) it is likely that the close association of FDCs with

nerve fibres in the intestinal wall facilitates the rapid spread of the TSE agent from PPs and mILFs to neighbouring enteric nerves.

#### **7.4 Relevance of work in this thesis to natural TSE diseases**

Accumulation of the TSE agent occurs in the lymphoid tissues of many cases of natural disease. Patients with vCJD have both PrP<sup>d</sup> (Hill *et al.*, 1999; Hilton *et al.*, 1998) and TSE infectivity (Bruce *et al.*, 2001) in lymphoid tissues such as the tonsils and appendix. A variety of different breeds of sheep with natural scrapie have PrP<sup>d</sup> in lymphoid tissues in the preclinical and clinical phases of disease (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2002; Heggebo *et al.*, 2000; Keulen *et al.*, 1999) and deer naturally affected by chronic wasting disease have accumulation of PrP<sup>d</sup> in tonsils and other lymphoid tissues (Fox *et al.*, 2006; Sigurdson *et al.*, 1999; Wild *et al.*, 2002). However, lymphoid tissue tropism is not a feature of all TSEs as PrP<sup>d</sup> is not detected in lymphoid tissues of cattle affected by BSE (Somerville *et al.*, 1997a), humans affected by sCJD (Hill *et al.*, 1999) and some strains of natural sheep scrapie (Ligios *et al.*, 2006). The ME7 strain of scrapie agent was used for the experiments described in this thesis because it is well studied and models the necessity for a lymphoid tissue replication phase which likely occurs in many natural TSE diseases. The data presented here demonstrate that draining lymphoid tissues are important for scrapie disease to become established in a murine model of the disease. This suggests that the early accumulation and replication of the TSE agent seen in draining lymphoid tissues in cases of natural TSE disease is also likely to play an important role in the successful establishment of disease.

The involvement of PPs in natural TSE disease has been shown by the detection of PrP<sup>Sc</sup> accumulation in the ileal PPs of 2 month old lambs in a naturally scrapie infected flock (Andreoletti *et al.*, 2000) and further supported by the demonstration of nerves in close association with FDCs in ovine PPs (Heggebo *et al.*, 2003). The involvement of PPs has also been shown by the presence of PrP<sup>d</sup> in the submucosal lymphatics around PPs within minutes of experimental inoculation of the sheep intestine with scrapie, and within the PPs and MLNs 30 days after inoculation (Jeffrey *et al.*, 2006). This pattern of PrP<sup>d</sup> accumulation in sheep is similar to that described in this thesis in mice, although data presented here suggests that the accumulation of PrP<sup>d</sup> in MLNs is not important for the establishment of disease. After experimental oral infection of mule deer fawns with CWD, PrP<sup>Sc</sup> was detected in PPs at early time points suggesting that PPs may also be involved in the pathogenesis of this disease (Sigurdson *et al.*, 1999). However, it is not known whether the involvement of PPs in natural disease is of crucial importance for neuroinvasion of the TSE agent. The data described in Chapter 4 of this thesis demonstrate the crucial importance of PPs for the neuroinvasion of scrapie in an experimental murine model of the disease, suggesting that the same may occur in animals with natural disease.

Results presented here raise the possibility of targeting a potential therapeutic treatment to the lymphoid tissues within the intestine (PPs or mLNs) for these diseases. For example, in the future it may become possible to target a treatment to lymphoid tissues in the intestine thus preventing the early replication and spread of the TSE agent to the CNS where irreparable and fatal damage is caused, whilst not



rendering the host completely immunocompromised as targeting a treatment to all lymphoid tissues may do. However, this is hampered by the lack of a pre-mortem diagnostic test and the rapid speed with which neuroinvasion occurs after oral exposure. The development of a vaccine would be an ideal way of controlling TSEs in the absence of a pre-mortem diagnostic or any therapeutic strategies to treat clinical cases. Several recent studies have investigated the possibility of developing a vaccine using either DNA (Fernandes-Borges *et al.*, 2006; Muller *et al.*, 2005) or recombinant prion protein (Bade *et al.*, 2006; Goni *et al.*, 2005; Ishibashi *et al.*, 2006). There has been encouraging success with the onset TSE disease being delayed after subsequent TSE challenge in all studies carried out. In this thesis, the absence of lymphoid tissues in the intestine was shown to prevent neuroinvasion after oral inoculation, thus a mucosal vaccine that prevents the TSE agent from reaching the lymphoid tissues in the intestine is likely to be successful in preventing subsequent disease from occurring.

## **7.5 The role of draining lymphoid tissues in other diseases**

The accumulation of infectious agent in lymphoid tissue prior to systemic spread is not a unique feature of TSEs. This has been observed in many other infectious diseases a few examples of which are discussed below.

### **7.5.1 Simian Immunodeficiency virus (SIV)**

The early pathogenesis of SIV has several features in common with TSEs. The involvement of draining lymphoid tissue prior to the involvement of other lymphoid tissue has been shown by the presence of SIV infected cells only in the iliac lymph

nodes 2 days after intra-vaginal inoculation of rhesus macaques, whereas infected cells were detected systemically by 5 days post inoculation (Spira *et al.*, 1996). After rectal inoculation of rhesus macaques with SIV, provirus was detected in the draining paracolic lymph nodes 7 days after inoculation but not seen in the non-draining axillary lymph nodes until 10 days after inoculation (Couedel-Courteille *et al.*, 1999). Similarly, after application of SIV particles to the tonsils of macaques replication of the virus was first observed in this lymphoid tissue prior to spread to other lymphoid tissues (Stahl-Hennig *et al.*, 1999). Thus these studies demonstrate, like TSEs, the lymphoid tissue responsible for the initial accumulation and replication of the infectious agent changes depending on route of exposure. It would be interesting to investigate the importance of the initial phase of SIV replication in draining lymphoid tissue in a similar way to the experiments described in this thesis for the investigation of scrapie. There are also parallels to be drawn between cells within lymphoid tissue responsible for the accumulation of the infectious SIV and TSE agents. As described elsewhere in this thesis (section 1.2.4) the attachment of the TSE agent to FDCs via complement components is important for the development of TSE disease. Similarly, studies have also shown that FDCs can harbour a reservoir of infectious HIV particles via interactions between HIV particles, complement components and complement receptors on FDCs (Stoiber *et al.*, 2001).

### **7.5.2 Venezuelan equine encephalitis virus (VEE)**

VEE is another disease which has some parallels to TSE disease. There is a distinct lymphoid replication phase of this virus prior to spread to the CNS where disease

including spongiform pathology is caused (Charles *et al.*, 2001; Gleiser *et al.*, 1962). However, unlike TSEs, this disease is spread by mosquitoes and predominantly affects horses and humans in Central and South America (Weaver *et al.*, 2004). A murine model of the disease has been established by the sub-cutaneous injection of VEE viral particles into the foot pad of mice. After this route of inoculation replication of viral particles is seen in the popliteal lymph node within four hours. Replication of the virus is not detected in non-draining lymphoid tissues until 12-24 hours after inoculation (Grieder *et al.*, 1995). This shows that initial drainage of the virus was to the local lymphoid tissue. It would be interesting to determine the importance of this initial replication phase in the popliteal lymph node performing similar experiments to the ones described in this thesis. However, when SCID mice were challenged with VEE all mice developed disease with an increased disease incubation period (Charles *et al.*, 2001). It is likely, therefore, that the same outcome would be observed in mice lacking the popliteal lymph nodes.

### **7.5.3 *Listeria monocytogenes***

A study on the early pathogenesis of *L. monocytogenes* infection after inoculation of ligated ileal loops in rats revealed initial targeting of cells in the PPs followed 6 hours later by the presence of infected cells in the MLNs (Pron *et al.*, 2001). As data presented here in chapter 4 revealed that in the absence of PPs the scrapie agent was unable to spread to MLNs after oral inoculation it would be interesting to investigate whether this is also the case with *L. monocytogenes* infection. If it were so, this would suggest that the transport of antigens from the intestine to the MLNs may be dependent on the presence of PPs or factors produced by PPs. After intra-peritoneal



inoculation with *L. monocytogenes* bacteria were detected in the mediastinal lymphocentrum (termed cranial mediastinal, tracheobronchal and caudal mediastinal lymph nodes in this thesis) prior to detection in the spleen and other non-draining lymph nodes (Marco *et al.*, 1992). This again demonstrates the initial spread of antigen to the local lymphoid tissue and that the site of initial accumulation of antigen changes depending on the route of inoculation.

#### **7.5.4 Other infectious diseases**

*Yersinia pestis* inoculated via the intra-tracheal route was found in mediastinal lymph nodes 24 hours after inoculation, but was not found in the spleen at this time point. 96 hours after inoculation *Y. pestis* was found in both the mediastinal lymph nodes and spleen demonstrating spread from draining lymphoid tissues to other lymphoid tissues (Bosio *et al.*, 2005). When mice were treated with clodronate to deplete antigen presenting cells (APCs) prior to inoculation a higher number of bacteria were detected in the spleen compared to untreated mice (Bosio *et al.*, 2005). This shows that trafficking of *Y. pestis* to the local lymphoid tissue is an active process to prevent early body wide dissemination. The immune response to *Herpes simplex* virus injected sub-cutaneously into the right ears of mice elicited an immune response characterised by the production of interferon- $\alpha/\beta$  in the draining lymph node of the right ear but not in that draining the left ear or any other lymphoid tissue (Riffault *et al.*, 1996). These studies suggest that the mechanism of antigen trafficking to the lymph node draining the site of inoculation has evolved to contain and deal with the infection locally to the site of inoculation so that pathogens and potentially harmful immune responses do not need to become bodywide. However, this mechanism is

detrimental to the host as far as many TSEs are concerned as it brings the infectious agent in contact with cells which may facilitate replication of the agent and support neuroinvasion.

## **7.6 Isolated lymphoid follicles**

Another important aim of this thesis was to characterise isolated lymphoid follicles, structures that have only recently been described in the murine small intestine (Hamada *et al.*, 2002), with a view to determining their potential role in scrapie agent neuroinvasion from the intestine. These structures have been classified into two types, mature and immature (Lorenz *et al.*, 2003). Here, the presence of FDCs in ILFs was found to correlate directly with ILF maturity. Further analysis revealed greater numbers of mILFs in the intestines of mice which lacked PPs compared to mice which contained PPs (Chapter 3). Indeed, the total area covered by FDCs in PP-deficient mice and control mice were found to be equivalent. These observations raised the possibility that neuroinvasion might also take place from FDCs within mILFs (Chapter 3). This was investigated in PP-deficient mice which contained numerous mILFs. These mice were found to be as susceptible to orally inoculated scrapie and develop disease in the same time frame (Chapter 4). Thus mILFs are identified in this thesis as a novel site of neuroinvasion from the small intestine.

The consumption of TSE agent contaminated food and prion protein genotype are important risk factors that affect TSE susceptibility (Goldmann *et al.*, 1994; Wadsworth *et al.*, 2004). Recent studies suggest that inflammation may be another factor, either enhancing uptake from mucosal sites (Thackray *et al.*, 2004) or

expanding the tissue distribution of these agents (Heikenwalder *et al.*, 2005; Ligios *et al.*, 2005; Seeger *et al.*, 2005). Results presented in this thesis have shown that mILFs can support neuroinvasion (Chapter 4). The formation and maturation status of ILFs can be modulated both by luminal bacterial flora (Fagarasan *et al.*, 2002; Lorenz *et al.*, 2003) and pathogenic micro-organisms (Little *et al.*, 2005) thus ILFs share features with the ectopically-induced germinal centres described above. It is possible to postulate, therefore, that neuroinvasion may also occur from lymphoid tissues induced during chronic inflammation, supporting the argument that inflammation is an important risk factor affecting TSE susceptibility. Mice genetically deficient in the anti-inflammatory cytokine IL-10 were found to succumb to scrapie disease much faster than WT counterparts (Thackray *et al.*, 2004). The effect of co-incident infection with a TSE agent and an inflammation inducing pathogen, which would more closely model a natural situation, has not been investigated. This could be done by infecting mice with, for example, *Eimeria* spp which induce an acute inflammatory response in the intestine (Roberts *et al.*, 1996), at the same time as administering the scrapie agent via the oral route. In the light of the evidence discussed above it would be expected that the scrapie disease incubation period in mice with co-incident inflammation and scrapie agent infection may be shortened.

Studies in this thesis and elsewhere have shown that  $LT\alpha_1\beta_2$  signalling through  $LT\beta R$  is vital for the formation of ILFs as  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice do not develop ILFs (Chapter 3; Lorenz *et al.*, 2003; McDonald *et al.*, 2005). The source of  $LT\alpha_1\beta_2$  for the formation of mILFs has been shown to be B lymphocytes (Lorenz *et al.*,



2003; McDonald *et al.*, 2005). mILF formation has also been found to be dependent on TNFR1 and intestinal flora as both TNFR1<sup>-/-</sup> mice and germ-free mice lack mILFs (Lorenz *et al.*, 2003). None of these are required for the formation iILFs, as iILFs are found in the intestines of germ-free mice, TNFR1<sup>-/-</sup> mice and LT $\alpha$ <sup>-/-</sup> mice grafted with LT-sufficient NK cells and T lymphocytes alone (Lorenz *et al.*, 2003; McDonald *et al.*, 2005). In the absence of Ig-isotype class switching and impaired IgA production in activation-induced cytidine deaminase (AID)-deficient mice the number of ILFs in the intestine is increased compared to control mice (Fagarasan *et al.*, 2002). This implies that inflammatory responses precipitated by a relative deficiency of intestinal IgA also stimulates the formation of ILFs. There is a general consensus that iILFs become mILFs given the right conditions (McDonald *et al.*, 2005; Newberry & Lorenz, 2005; Taylor & Williams, 2005). However, there is no direct evidence that iILFs are a precursor to mILFs nor is there any evidence against iILFs and mILFs representing the terminal stages of discrete pathways of development. Recent studies have shed some light on this issue. One study coined the term solitary intestinal lymphoid tissues (SILT) to describe all isolated lymphoid aggregations in murine small intestine (Pabst *et al.*, 2006). These aggregations include iILFs, mILFs and also cryptopatches which are aggregations found in intestinal crypts. Cryptopatches contain haematopoietic precursor cells and were originally thought to be extra-thymic sites of T-lymphocyte development (Saito *et al.*, 1998; Suzuki *et al.*, 2000). More recent work has challenged this idea and suggested the predominant cell population within cryptopatches may adult counterparts of foetal lymphoid tissue initiating cells (LTICs) and therefore cryptopatches are precursors of ILFs (Eberl & Littman, 2004). The total number of

SILT in germ-free mice was the same compared to control mice although in germ-free mice SILT were of a predominantly small immature phenotype (Pabst *et al.*, 2006). However, when germ-free mice were colonised with commensal bacteria the SILT became progressively larger demonstrated a more mature phenotype similar to that found in control mice. This led the authors to propose that SILT are a secondary lymphoid tissue whose maturation status depends on factors in the local environment (Pabst *et al.*, 2006). These findings suggest that iILFs can mature into mILFs and also that cryptopatches are part of the same spectrum of lymphoid aggregations.

The study of ILFs in this thesis has also revealed the presence of IgA in the intestines of mice containing mILFs and lacking PPs and MLNs. IgA was absent from mice lacking PPs, MLNs and mILFs despite the presence of FDCs in remaining lymphoid tissues in these mice (Chapter 3). This suggested that IgA synthesis in the intestines of mice with no PPs or MLNs was dependent on the presence of mILFs. These observations are consistent with the work of others which showed that after infection with *Salmonella typhimurium*, specific IgA was present in intestines of mice which had mILFs, but lacked PPs or MLNs (Lorenz & Newberry, 2004). Likewise, cells isolated from the mILFs of SRBC immunised mice have been shown to be capable of making SRBC-specific IgA upon restimulation (Lorenz & Newberry, 2004). Collectively, these data suggest that mILFs are capable of producing IgA, and, therefore, display similar functionality to other gut-associated lymphoid tissues. This is consistent with the hypothesis that the increased number of mILFs seen in mice lacking PPs is to provide immunological protection for the intestines in the absence of PP function. As deficiency of intestinal IgA is thought to be a trigger for ILF

formation (Fagarasan *et al.*, 2002) it is logical that mILFs should produce IgA in order to compensate for this deficiency. Two recent studies, however, have suggested that ILFs are not essential for the induction of intestinal IgA responses. In this study, mice which lacked PPs and MLNs through combined *in utero* TNFR1- and LT $\beta$ R-signalling blockade failed to induce an IgA response after oral immunisation with tetanus toxoid (Yamamoto *et al.*, 2004). The maturation status of ILFs in this study was not recorded and as TNFR1-stimulation is essential for mILF formation (Lorenz *et al.*, 2003) it is plausible that ILF maturation was significantly impaired in these mice leading to a failure to induce an IgA response. Another study showed near normal levels of IgA in the intestines of CD40<sup>-/-</sup> mice despite a failure to detect germinal centres or Ig class-switching in the PPs, MLNs or ILFs (Bergqvist *et al.*, 2006). This led the authors to postulate that the induction of intestinal IgA production occurs outwith the gut-associated lymphoid tissues (GALT), although they do not suggest a location for this.

## **7.7 Conclusion**

Data presented here show that lymphoid tissues local to the site of inoculation of the ME7 scrapie agent are important for efficient neuroinvasion to occur. The relative importance of the draining lymphoid tissues varies depending on the route of inoculation. After oral inoculation PPs within the intestine are crucial, as in their absence neuroinvasion does not occur. MLNs are not important after oral inoculation as in the absence of PPs and presence of MLNs disease did not result, demonstrating that lymphoid tissues within the intestine itself were required (Chapter 4). In the absence of PPs an increased number of mature ILFs develop within the



intestine. Mature ILFs resemble PPs but are very much smaller and occur as single follicles rather than being an aggregation of several follicles. The increased number, size and maturity of ILFs in the intestines of PP-deficient mice results in an equal number of FDCs being present in the intestines of PP-deficient and control mice (Chapter 3). When inoculated orally with the scrapie agent the increased number, size and maturity of ILFs in the intestines of PP-deficient mice results in PP-deficient mice being as susceptible as control mice to scrapie disease (Chapter 4). After inoculation via scarification of the thigh, mice which lacked the draining inguinal lymph node were less susceptible to scrapie challenge than immunocompetent controls (Chapter 5). After i.p. inoculation  $LT\alpha^{-/-}$  mice were less susceptible and had delayed onset of disease compared to WT mice, whereas  $LT\beta^{-/-}$  mice were as susceptible and developed disease in the same timeframe as WT mice. This raised the possibility that lymphoid tissue present in  $LT\beta^{-/-}$  mice and absent from  $LT\alpha^{-/-}$  mice (or, theoretically, a cell type relying on  $LT\alpha$  expression by a stromal derived cell) were important in supporting neuroinvasion (Chapter 6). Taken together these results suggest that the initial accumulation of the agent in lymphoid tissue draining the site of inoculation is important for scrapie agent neuroinvasion. Understanding the earliest stages of scrapie pathogenesis will help in the development of therapeutic strategies for TSE diseases and further general understanding of the lymphatic vasculature and the initial uptake of foreign particles.

## **Appendix 1: Publication List**

All publications are included at the end of this thesis.

**Glaysheer B. R. & Mabbott N.A.** (2007). Isolated lymphoid follicle maturation induces the development of follicular dendritic cells. *Immunology*. 120: 336-344. (Included with kind permission of *Immunology* and Blackwell Publishing.)

**Glaysheer B. R. & Mabbott N.A.** (2007). Role of GALT in scrapie agent neuroinvasion from the intestine. *Journal of Immunology*. 178: 3757-3766. (Copyright 2007. The American Association of Immunologists, Inc.)

**Glaysheer B. R. & Mabbott N.A.** (2007). Role of the draining lymph node in scrapie agent transmission from the skin. *Immunology Letters*. 109: 64-71. (Copyright 2007. European Federation of Immunological Societies. With permission from Elsevier.)

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# Isolated lymphoid follicle maturation induces the development of follicular dendritic cells

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## Summary

Isolated lymphoid follicles (ILFs) are recently identified lymphoid structures in the small intestine with features similar to Peyer's patches (PPs). Using immunohistochemistry we characterized the composition of ILFs in the small intestines of immunocompetent mice and of mice that lacked PPs as a result of either genetic deficiency of lymphotoxin or temporary *in utero* lymphotoxin- $\beta$  receptor-signalling blockade. We showed that although both immature and mature ILFs were present in the intestines of immunocompetent mice, PP-deficiency induced a significantly greater number of mature ILFs. We found that in addition to B-lymphocyte-containing germinal centres, mature ILFs also possessed large networks of follicular dendritic cells (FDCs). These features were not detected within immature ILFs. Indeed, the presence of FDCs could be used to reliably distinguish ILF maturity. Further analysis revealed that the area occupied by the FDCs within mature ILFs was substantial. The total area occupied by FDCs in all the mature ILFs in mice lacking PPs was equivalent to the total area occupied by FDCs in all the PPs and the few mature ILFs in immunocompetent mice. Based on these data we reasoned that in the absence of PPs, mature ILFs are important inductive sites for intestinal immune responses. Indeed, in mice that lacked PPs, ILF maturation coincided with a restoration of faecal immunoglobulin A levels to values that were comparable to those found in immunocompetent mice. Taken together, these data imply that the induction of germinal centres and FDC networks within mature ILFs in response to PP deficiency provides an important compensatory mechanism.

**Keywords:** follicular dendritic cells; intestine; isolated lymphoid follicles; lymphotoxin; Peyer's patches

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## Introduction

The gastrointestinal tract is a major interface between host and potential pathogenic micro-organisms. Highly organized lymphoid structures, such as Peyer's patches (PPs) and diffusely distributed effector cells within the epithelium and lamina propria, help to protect the mammalian gastrointestinal tract against infection by microbial pathogens. Secretory immunoglobulin A (IgA) is an important component of the intestinal immune response and is synthesized by plasma cells in the lamina propria.<sup>1</sup> In the intestine, IgA isotype class switching occurs only in organized lymphoid structures such as

PPs<sup>2</sup> but antigen-specific IgA responses have been demonstrated in mice that are deficient in PPs.<sup>3</sup> As a consequence, novel lymphoid clusters, termed isolated lymphoid follicles (ILFs), were identified along the antimesenteric wall of the mucosa of the small intestine.<sup>4</sup> Isolated lymphoid follicles are much smaller than PPs and comprise a single B-lymphocyte follicle with a germinal centre (GC) and an overlying M-cell-containing epithelium similar to the follicle-associated epithelium of PPs.<sup>4</sup> Isolated lymphoid follicles are distributed throughout the small and large intestines of mice<sup>4–6</sup> and have also been demonstrated in the intestines of rabbits,<sup>7</sup> rats,<sup>8</sup> guinea-pigs<sup>9</sup> and humans.<sup>10</sup>



Interactions between the membrane bound lymphotoxin (LT)  $\alpha_1\beta_2$  heterotrimer and the LT $\beta$  receptor (LT $\beta$ R) during gestation are crucial for the formation of PPs and other secondary lymphoid tissues. Accordingly, mice that are deficient in LT $\alpha$ , LT $\beta$  or LT $\beta$ R lack PPs and most other lymph nodes.<sup>11</sup> Likewise, temporary *in utero* LT $\beta$ R-blockade during the period of embryonic lymphoid tissue formation also blocks the development of PPs and certain lymph nodes.<sup>12</sup> The factors required for ILF development share similarities with those required for PP development but there are key differences. For example, stimulation via LT $\beta$ R is also important for the development of ILFs because they are absent in mice that are deficient in LT or LT $\beta$ R.<sup>4,13</sup> However, unlike PP formation, ILF formation occurs postnatally because *in utero* LT $\beta$ R-signalling blockade does not inhibit ILF formation, and their development in adult LT-deficient mice can be restored by reconstitution with LT-expressing bone marrow.<sup>4,13,14</sup>

Follicular dendritic cells (FDCs) reside within B-lymphocyte follicles in GCs and are specialized to trap and retain antigen on their surfaces. Antigen trapped on the surface of FDCs is considered to promote immunoglobulin isotype class switching and affinity maturation of naive IgM<sup>+</sup> B lymphocytes.<sup>15–19</sup> Consistent with their role as important sites for the generation of IgA responses,<sup>2</sup> PPs contain all the necessary cellular components required to generate IgA-committed B lymphocytes including B-lymphocyte follicles, with GCs, T cells and FDC networks. The ILFs appear structurally and functionally similar to PPs<sup>4</sup> and their inductive nature implies that they are a complementary system for the generation of intestinal IgA responses.<sup>13</sup> In this study we demonstrate that mature ILFs also contain large FDC networks. The presence of FDC networks within gut-associated lymphoid tissues is considered important for the induction of intestinal IgA responses.<sup>20</sup> Here, the induction of FDC maturation within ILFs of mice lacking PPs and mesenteric lymph nodes (MLNs) coincided with a restoration of faecal IgA to levels comparable with those found in immunocompetent mice. Therefore, our data suggest that the FDC networks within ILFs provide the necessary microenvironment to promote efficient interaction between luminal derived antigens and B lymphocytes to stimulate the generation of effective IgA responses.

## Materials and methods

### Mice

Both LT $\alpha$ <sup>−/−</sup> mice<sup>21</sup> and LT $\beta$ <sup>−/−</sup> mice<sup>22</sup> were obtained from B & K Universal Ltd (Hull, UK) and were maintained on a C57BL/6 background. Age-matched and sex-matched C57BL/6 mice were used as immunocompetent wild-type

(WT) controls in the studies using LT $\alpha$ <sup>−/−</sup> mice and LT $\beta$ <sup>−/−</sup> mice. Severe combined immunodeficiency (SCID) mice were maintained on a 129/Ola background.<sup>23</sup>

### $\gamma$ -irradiation and bone marrow reconstitution

Bone marrow from the femurs and tibias of immunocompetent C57BL/6 WT mice was prepared as a single-cell suspension ( $3 \times 10^7$  to  $4 \times 10^7$  viable cells/ml) in Hank's balanced salt solution (Life Technologies, Paisley, UK). Recipient adult (6–8 weeks old) LT $\alpha$ <sup>−/−</sup> mice, LT $\beta$ <sup>−/−</sup> mice and C57BL/6 mice were  $\gamma$ -irradiated (950 rads) and 24 hr later were reconstituted with 0.1 ml bone marrow by injection into the tail vein.

### PP-deficient mice

To create progeny mice that were deficient in PPs, timed pregnant C57BL/Dk mice were given a single intravenous injection of 100  $\mu$ g of a fusion protein containing the soluble LT $\beta$ R domain linked to the Fc portion of human IgG1 (LT $\beta$ R-Ig<sup>24</sup>) on day 11.5 of gestation.

### Immunohistochemical and immunofluorescent analysis

Spleens were snap-frozen at the temperature of liquid nitrogen. Small intestine from each mouse was divided into three roughly equal parts, gently squeezed to remove the gut contents, coiled, embedded in Tissue-Tek<sup>®</sup> OCT Compound<sup>™</sup> (Bayer Plc., Newbury, UK) and snap frozen at the temperature of liquid nitrogen. Serial frozen sections (10  $\mu$ m thickness) were cut on a cryostat.

Follicular dendritic cells were visualized by staining with 8C12 monoclonal antiserum to detect CR1 (CD35; BD Biosciences PharMingen, Oxford, UK) or 7G6 monoclonal antiserum to detect CR2/CR1 (CD21/CD35; BD Biosciences PharMingen). Complement components C3 and C4 were detected using RMC7H8 (Connex, Martinsreid, Germany) and FDC-M2 (AMS Biotechnology, Oxon, UK) monoclonal antisera, respectively. B lymphocytes were detected using B220 monoclonal antiserum to detect CD45R (Caltag, Towcester, UK), or biotin-conjugated peanut agglutinin (Sigma, Poole, UK) to detect GC B lymphocytes. T lymphocytes were detected using CD3 $\epsilon$ -specific monoclonal antiserum 2C11 (BD Biosciences Pharmingen).

For light microscopy, following the addition of primary antibody, biotin-conjugated species-specific secondary antibodies (Stratech, Soham, UK) were applied followed by alkaline phosphatase coupled to the avidin/biotin complex (Vector Laboratories, Peterborough, UK). Vector Red (Vector Laboratories) was used as a substrate, and sections were counterstained with haematoxylin to distinguish the cell nuclei. For fluorescent



microscopy, following the addition of primary antibody, species-specific secondary antibodies coupled to Alexa dyes (Invitrogen, Paisley, UK) 488 (green) or 594 (red) were used. Sections were mounted in fluorescent mounting medium (Dako, Ely, UK) and examined using a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK).

#### *ILF enumeration and morphometric analysis of FDC networks*

Entire small intestines were divided into three equal parts, coiled, fixed in paraformaldehyde and embedded in paraffin wax. Serial sections (10 µm thickness) were deparaffinized and pretreated with Target Retrieval Solution (DAKO Ltd, Ely, UK). Sections were immunostained with antisera specific for CD45R or CR2/CR1 as described above and examined using a confocal microscope. The total number of B-lymphocyte-containing ILFs in the entire small intestine of each mouse analysed was counted microscopically using CD45R expression for identification. The maturity of each ILF was determined according to size and the location and density of component cells as previously described.<sup>13</sup> An image of each mature ILF was captured and the area covered by FDCs (CR2/CR1<sup>+</sup> CD45R<sup>-</sup> cells) was measured using IMAGE PRO PLUS software (Media Cybernetics, Wokingham, UK).

#### *Measurement of faecal IgA*

Faecal pellets were collected from individual mice and faecal IgA levels were compared by enzyme-linked immunosorbent assay. Briefly, 10% faecal homogenates were prepared in carbonate-bicarbonate coating buffer (Sigma) and centrifuged for 10 min at 12 000 g. Supernatant was removed and absorbed to flat-bottomed, high-binding microwell plates (Costar®, High Wycombe, UK) for 18–24 hr at 4°. Plates were then blocked with 0.01 M phosphate-buffered saline (pH 7.5) containing 5% bovine serum albumin. Bound IgA was detected using alkaline phosphatase-conjugated goat anti-mouse IgA (Southern Biotechnology, Birmingham, AL). Bound alkaline phosphatase activity was measured by incubation with *p*-nitrophenyl phosphate liquid substrate (Sigma). Optical density was measured at 450 nm using a V-max kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Faecal pellets from C57BL/6 WT mice, C57BL/Dk control mice and SCID mice were used as reference controls.

#### *Statistical analysis*

Data are presented as mean ± standard error (SE). Significant differences between samples in different groups were sought by one-way analysis of variance (ANOVA).

## Results

### **Temporary *in utero* LTβR-signalling blockade induces the maturation of ILFs in progeny mice**

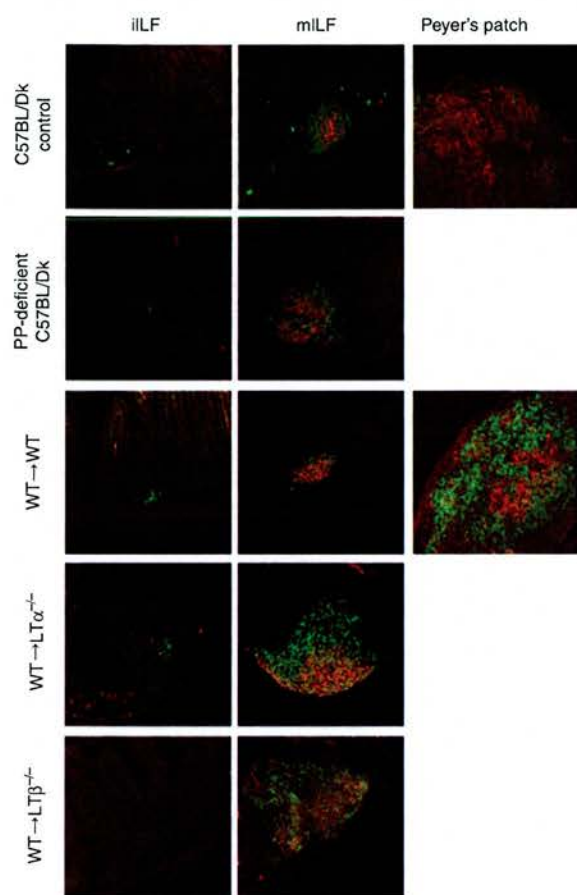
To temporarily blockade the LTβR-signalling pathway *in utero*, pregnant C57BL/Dk mice were given a single intravenous injection of 100 µg LTβR-Ig on day 11.5 of gestation. Consistent with previous reports,<sup>12</sup> the intestines from age-matched, untreated, immunocompetent C57BL/Dk (control) mice contained approximately six PPs per mouse ( $n = 11$ ), whereas no PPs were detected in any of the small intestines taken from progeny mice treated *in utero* with LTβR-Ig ( $n = 12$ ). Microscopic analysis revealed the presence of both PPs and ILFs in the intestines of untreated C57BL/Dk (control) mice (Fig. 1), whereas only ILFs were found in the intestines of mice treated *in utero* with LTβR-Ig (termed PP-deficient C57BL/Dk mice; Fig. 1).

The maturity of ILFs has previously been defined according to size and the location and density of the component cells.<sup>13</sup> Immature ILFs (iILFs) comprise loosely clustered CD45R<sup>+</sup> (B220) cells located at the base of a villus. Mature ILFs (mILFs), in contrast, are well-organized nodular structures, of similar width to or greater than the width of one villus, occurring singly or in groups of two, with a GC and an overlying dome typical of the follicle-associated epithelium of a PP. Using these criteria, double immunostaining for B lymphocytes (CD45R<sup>+</sup> cells) and FDCs (CR2/CR1<sup>+</sup> cells) on the same tissue section revealed that the presence of FDCs (CR2/CR1<sup>+</sup> CD45R<sup>-</sup> cells) could also be reliably used to distinguish ILF maturation status. Our analysis showed that all mILFs contained FDCs, whereas iILFs did not (Fig. 1). The FDCs characteristically trap and retain antigens in the form of immune complexes composed of antigen, antibody and/or complement components, storing them in native form on the cell surface for long periods.<sup>25</sup> Immune complex retention on FDCs is highly dependent on complements and the expression of CR1 (CD35) and CR2 (CD21). Complement components C3 and C4, in association with FDCs expressing CR1 and CR2, were detected within mILFs (Fig. 2) but not within iILFs (not shown). Taken together, these data clearly demonstrate that mILFs in the intestines of PP-deficient C57BL/Dk mice contain immune complex-trapping FDCs.

### **FDCs in mILFs compensate for PP deficiency**

We next assessed the number of ILFs in the intestines of PP-deficient mice and control mice, and also compared the size of the FDC networks within them. Entire small intestines were double immunostained with monoclonal antisera specific for B lymphocytes (CD45R<sup>+</sup> cells) and CR2/CR1-expressing FDCs. Sections were examined





**Figure 1.** Detection of B lymphocytes and FDCs within ILFs and PPs in the small intestine. Entire small intestines were examined from each mouse group and representative images are shown. Sections were stained with the CD45R-specific monoclonal antiserum B220 to detect B lymphocytes (green) and 7G6 monoclonal antiserum to detect CR2/CR1 (CD21/35) expressing FDCs (red). In each mouse group analysed, FDCs were absent within the B-lymphocyte aggregations of immature isolated lymphoid follicles (iILFs; left column), whereas both B lymphocytes and FDCs were detected within all mature ILFs (mILFs; middle column). Original magnification  $\times 200$ .

microscopically, the ILFs were counted, their maturation status was recorded and the area within them occupied by FDCs was measured. The total number of ILFs in the small intestine of PP-deficient mice was found to be significantly greater than the number in C57BL/Dk (control) mice ( $P = 0.001$ ; Fig. 3a). Approximately four-fold more ILFs were detected in the intestines of PP-deficient mice than in those of control mice. Although the small intestines of both groups of mice were found to contain both mLfFs and iILFs (Fig. 1), a significantly greater proportion of the ILFs in PP-deficient mice were mature when compared to those in control mice (46% and 15%, respectively;  $P = 0.001$ ; Fig. 3b).

Next, we measured the area of the FDC networks within the mLfFs in PP-deficient mice and those within both the PPs and mLfFs in control mice. We found that the area occupied by FDCs within individual mLfFs from PP-deficient mice was significantly larger than the area they occupied in mLfFs from control mice ( $P = 0.001$ ; Fig. 3c). Furthermore, the total area occupied by FDCs in all the mLfFs in PP-deficient mice was equivalent to the total area occupied by FDCs in all the PPs and the few mLfFs found in control mice ( $P = 0.572$ ; Fig. 3d).

#### LT-expressing bone marrow-derived cells induce the development of ILFs in LT-deficient mice

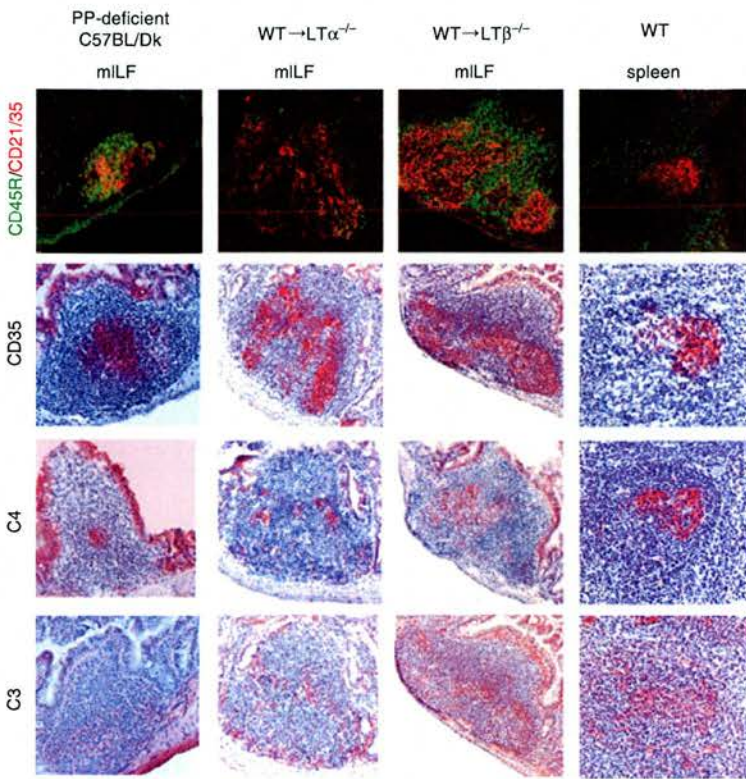
Both PPs and ILFs are absent in the intestines of LT-deficient mice because signalling via the LTβR is required for their formation.<sup>11,13,26</sup> However, reconstitution of adult LTα<sup>-/-</sup> mice<sup>13</sup> or LTβ<sup>-/-</sup> mice with LT-expressing WT bone marrow (termed WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice, respectively) restored the formation of ILFs (Figs 1 and 4), but not the development of PPs (data not shown). When assessed 105 days after WT bone marrow reconstitution, the total number of ILFs in the intestines of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice was equivalent to the number detected in WT→WT mice (Fig. 4a).

#### Mature ILFs in WT bone marrow reconstituted LT-deficient mice contain FDCs

We next examined the ILFs in intestines from WT→WT mice, WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice in greater detail. Entire small intestines were double immunostained with monoclonal antisera specific for B lymphocytes (CD45R<sup>+</sup> cells) and CR2/CR1-expressing FDCs. Sections were examined microscopically, the ILFs were counted, their maturation status was recorded and the area within them occupied by FDCs was measured. Although both iILFs and mLfFs were detected in the intestines of each group of mice (Fig. 1), a significantly greater proportion of the ILFs in WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice were mature when compared to those in WT→WT mice ( $P = 0.028$ ,  $n = 6$ , and  $P = 0.002$ ,  $n = 5$ , respectively, when compared to WT→WT mice; Fig. 4b).

Consistent with data from the analysis of PP-deficient mice, all the mLfFs in the intestines of WT→LTα<sup>-/-</sup> mice, WT→LTβ<sup>-/-</sup> mice and WT→WT mice contained functional FDC networks (Fig. 2). When the area of these FDC networks was measured, those within the mLfFs of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice were found to be significantly larger than those of WT→WT mice ( $P = 0.009$ ,  $n = 6$ , and  $P = 0.026$ ,  $n = 5$ , respectively, when compared to WT→WT mice,  $n = 6$ ; Fig. 4c). In accordance with data from PP-deficient C57BL/Dk mice, the total area occupied by FDCs in all the mLfFs in





**Figure 2.** Immunohistochemical characterization of FDCs within mILFs. Sections of small intestine or spleen (far right column) were stained with CD45R-specific monoclonal anti-serum B220 to detect B lymphocytes (top row, green), CR2/CR1 (CD21/35)-specific (top row, red) or CR1 (CD35)-specific monoclonal antisera to detect FDCs (second row, red) and monoclonal antisera specific for complement components C4 (third row, red) and C3 (bottom row, red). Complement components in association with CR1- and CR2-expressing FDCs were detected in both mILFs and the spleen. Original magnification  $\times 200$ .

WT $\rightarrow$ LT $\alpha^{-/-}$  mice or WT $\rightarrow$ LT $\beta^{-/-}$  mice was equivalent to the total area occupied by FDCs in all the PPs and the mILFs found in WT $\rightarrow$ WT mice ( $P = 0.099$ ,  $n = 6$ , and  $P = 0.162$ ,  $n = 5$ , respectively, when compared to WT $\rightarrow$ WT mice; Fig. 4d).

#### Mature ILFs contain T lymphocytes and GC B lymphocytes

Further immunohistochemical analysis revealed that GC B lymphocytes (peanut agglutinin-positive cells) were readily detected in the mILFs of all five groups of mice and were rarely detected in iILFs (Fig. 5a). Although ILFs lack the interfollicular T-lymphocyte regions of PPs, CD3-positive T lymphocytes were detected within both iILF and mILFs, and appeared to be more frequent in mILFs (Fig. 5b).

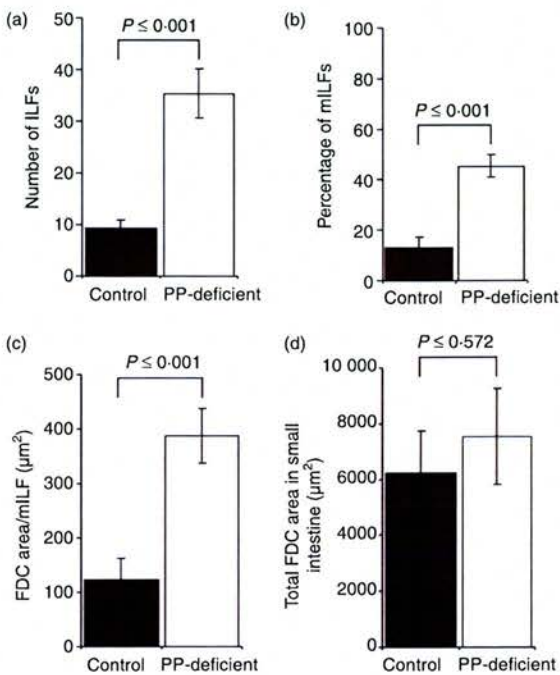
#### Enhanced intestinal IgA synthesis in the presence of mILFs

The finding that mILFs contain GC B lymphocytes and large FDC networks suggests that mILFs have the potential to function as inductive sites for mucosal immune responses. To assess this potential, we next compared the levels of IgA secreted into the intestines of mice lacking PPs and/or ILFs. Coincident with the absence of both PPs and mILFs, IgA levels in the faeces of LT $\alpha^{-/-}$  mice and

LT $\beta^{-/-}$  mice were substantially lower than those in the faeces of immunocompetent WT mice (Fig. 6a). Similarly, IgA was undetectable in the faeces of highly immunodeficient SCID mice. In contrast, the induction of ILF maturation in WT $\rightarrow$ LT $\alpha^{-/-}$  mice and WT $\rightarrow$ LT $\beta^{-/-}$  mice (Fig. 4) coincided with a significant increase in intestinal IgA synthesis ( $P = 0.05$ ,  $n = 4$ , and  $P = 0.020$ ,  $n = 4$ , respectively, when compared to unreconstituted LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice) to levels equivalent to those in both WT mice and WT $\rightarrow$ WT mice (Fig. 6a). Similarly, in PP-deficient C57BL/Dk mice which had no PPs but which had significantly increased numbers of mILFs (Fig. 3), faecal IgA levels were equivalent to those found in C57BL/Dk (control) mice ( $P = 0.245$ ,  $n = 4$ ; Fig. 6b). The absence of the MLNs in WT $\rightarrow$ LT $\alpha^{-/-}$  mice did not adversely affect faecal IgA levels because they were equivalent to those of WT mice, WT $\rightarrow$ WT mice and WT $\rightarrow$ LT $\beta^{-/-}$  mice, which possess them. Taken together these results suggest that mILFs are important inducible components of the mucosal immune system.

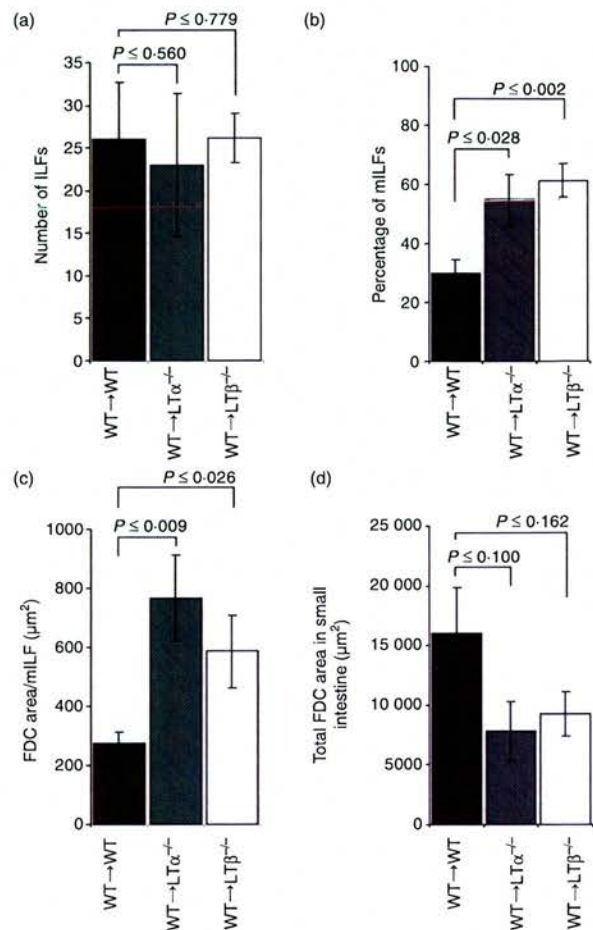
#### Discussion

The composition of ILFs in the murine small intestine was investigated. We found that although both iILFs and mILFs were present in the intestines of immunocompetent mice, a significantly greater number of mILFs was induced in a LT-dependent manner in the absence of



**Figure 3.** Enumeration of ILFs and FDC area in the small intestines of PP-deficient C57BL/Dk mice and immunocompetent C57BL/Dk (control) mice. For each mouse group, sections were stained with B-lymphocyte- (CD45R) and FDC- (CD21/35) specific antisera and the entire small intestine was examined microscopically. The number of ILFs was counted and the area occupied by FDCs was measured. (a) Intestines from PP-deficient mice contained significantly more (approximately four-fold more) ILFs than control mice. (b) Intestines from PP-deficient mice were found to have a significantly higher percentage of mLFs (46%) when compared to control mice (13%). (c) The area occupied by FDCs in individual mLFs from PP-deficient mice was significantly larger than those in mLFs from control mice. (d) The total area covered by FDCs in the mLFs in PP-deficient C57BL/Dk mice was equivalent to the total area covered by FDCs from all the PPs and mLFs in control mice. Each bar represents the mean  $\pm$  SE for each group of mice ( $n = 12$  for PP-deficient mice,  $n = 11$  for control mice).

PPs. Immunohistochemical analysis revealed that the presence of functional FDCs could also be used to reliably distinguish ILF maturity: all mLFs contained large FDC networks whereas none were detected within iILFs. The total area occupied by FDCs in all the mLFs in mice lacking PPs was substantial and was equivalent to the total area occupied by FDCs in all the PPs and the few mLFs found in immunocompetent mice. These data imply that the induction of GC and FDC network formation within mLFs in response to PP deficiency provides a mechanism to compensate for the absence of PPs. Indeed, in mice that lacked PPs, ILF maturation coincided with a restoration of faecal IgA levels to those comparable to immunocompetent mice.



**Figure 4.** Enumeration of ILFs and FDC area in the small intestines of WT→WT mice, WT→LT $\alpha^{-/-}$  mice and WT→LT $\beta^{-/-}$  mice 105 days after reconstitution with WT bone marrow. For each mouse group, sections were stained with B-lymphocyte- (CD45R) and FDC- (CD21/35) specific antisera and the entire small intestine was examined microscopically. The number of ILFs was counted and the area occupied by FDCs was measured. (a) Similar numbers of ILFs were found in WT→WT mice, WT→LT $\alpha^{-/-}$  mice and WT→LT $\beta^{-/-}$  mice. (b) However, WT→LT $\alpha^{-/-}$  and WT→LT $\beta^{-/-}$  mice were found to have a significantly higher percentage of mLFs when compared to WT→WT mice. (c) The area occupied by FDCs in individual mLFs from WT→LT $\alpha^{-/-}$  mice and WT→LT $\beta^{-/-}$  mice was significantly larger than those in WT→LT $\alpha^{-/-}$  mice. (d) The total area covered by FDCs in all the mLFs in WT→LT $\alpha^{-/-}$  mice or WT→LT $\beta^{-/-}$  mice was equivalent to the total area covered by FDCs in all the PPs and mLFs in WT→WT mice. Each bar represents the mean  $\pm$  SE for each group of mice ( $n = 4$  for each group).

The formation of iILFs is dependent upon stimulation from LT-expressing lymphocytes which interact with LT $\beta$ R-expressing stromal cells in the intestine and induce the formation of clusters of B220<sup>+</sup> cells at the base of a villus. This initial stimulus can be provided by LT-expressing B lymphocytes, T lymphocytes or NK cells. However,



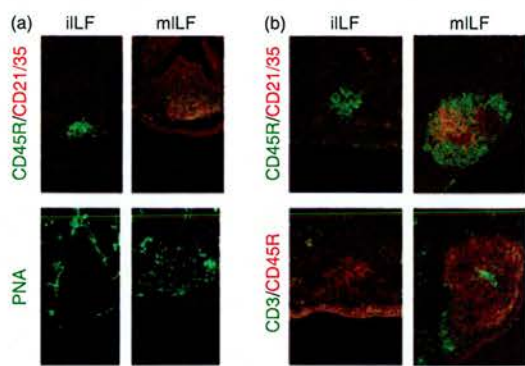


Figure 5. Immunohistochemical detection of (a) GC B lymphocytes and (b) T lymphocytes within ILFs. Entire small intestines were examined from PP-deficient C57BL/Dk mice and representative images are shown. Sections were stained with B lymphocyte- (CD45R, green) and FDC- (CD21/35, red) specific antisera to distinguish between iILFs and mILFs (upper row). (a) To detect GC B lymphocytes, adjacent sections were stained with peanut agglutinin (green) (lower row). Peanut agglutinin-binding GC B lymphocytes were detected only in mILFs. (b) To detect T lymphocytes, adjacent sections were stained with CD3-specific monoclonal antiserum (green) (lower row). T lymphocytes were found in mILFs, whereas few or none were found in iILFs. Original magnification  $\times 200$ .

in response to exogenous stimuli, LT-expressing B lymphocytes trigger the progression of iILFs into mILFs resembling lymphoid nodules containing a single B-lymphocyte follicle of predominantly B2-B lymphocytes with a GC and a follicle-associated epithelium containing M cells.<sup>2,13,14</sup> In this study we also demonstrate that LT-expressing bone marrow-derived cells also induce the differentiation of large FDC networks within mILFs.

Follicular dendritic cells trap and retain antigens on their surfaces through interactions between complement components and cellular CR1 and CR2.<sup>27,28</sup> In our morphometric analysis studies described above anti-CR2/CR1 antiserum was used to detect FDCs as they characteristically express these receptors at high levels.<sup>27</sup> However, in the mouse CR1 and CR2 are also expressed on B lymphocytes, but at lower levels than on FDCs. Thus CR2/CR1<sup>+</sup> B lymphocytes were excluded from our assessments of FDC size and our calculations were based only on CR2/CR1<sup>+</sup> CD45R<sup>-</sup> cells. Parallel studies also demonstrated complement components C3 and C4, in association with large networks of CR1-expressing and CR2/CR1-expressing cells (Fig. 2). These data confirm that the CR2/CR1<sup>+</sup> CD45R<sup>-</sup> cells identified within mILFs were functional FDCs capable of trapping and retaining C3-bearing and C4-bearing immune complexes.<sup>27,29</sup>

Several lines of evidence support the conclusion that antigen trapped on the surface of FDCs promotes immunoglobulin-isotype class switching and affinity maturation of naive IgM<sup>+</sup> B lymphocytes.<sup>15-19</sup> Follicular dendritic cells require important cytokine stimulation from lymphocytes,

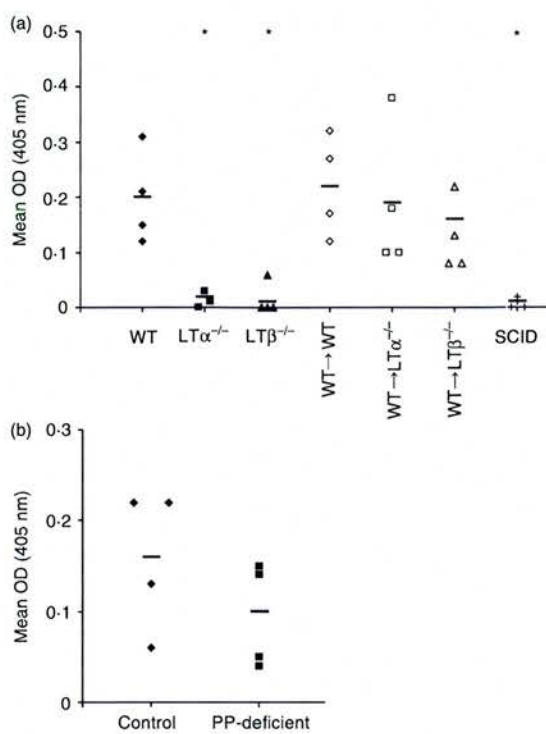


Figure 6. The presence of mILFs in mice lacking PPs and MLNs correlates with enhanced IgA synthesis. Faecal IgA levels were determined by enzyme-linked immunosorbent assay and are expressed as the mean optical density (OD) at 450 nm for triplicate samples from individual mice. The horizontal bar represents mean OD for each experimental group of mice ( $n = 4$ ). (a) In contrast to WT mice, faecal IgA levels in both LT $\alpha^{-/-}$  and LT $\beta^{-/-}$  mice were extremely low, but increased significantly after reconstitution with WT bone marrow. (b) C57BL/Dk (control) mice and PP-deficient C57BL/Dk mice were found to have equivalent levels of faecal IgA. SCID mice (+) were used as negative controls. \*Indicates significant difference from WT mice ( $P = 0.05$ ).

such as membrane LT $\alpha_1\beta_2$  and tumour necrosis factor- $\alpha$ , to maintain their differentiated state.<sup>30</sup> Hence, in the absence of LT $\beta$ R-stimulation in LT $\alpha^{-/-}$  mice, LT $\beta^{-/-}$  mice and LT $\beta$ R<sup>-/-</sup> mice, FDC networks and GCs do not develop and immunoglobulin-isotype class switching is dramatically impaired. However, reconstitution of LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice with WT bone marrow induces the development of FDC networks and GCs in the spleen, supporting effective immunoglobulin-isotype class switching.<sup>15-18,31</sup> Secretory IgA is a major component of the mucosal immune system and provides an important first line of defence against infection by microbial pathogens across mucosal surfaces.<sup>1</sup> In this study, coincident with the lack of FDCs and organized gut-associated lymphoid tissues, IgA levels were undetectable in the faeces of LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice. However, reconstitution of LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice with WT bone marrow (WT $\rightarrow$ LT $\alpha^{-/-}$  mice and WT $\rightarrow$ LT $\beta^{-/-}$  mice, respectively), induced the



formation of ILFs and the maturation of FDCs and GCs within them, and coincided with an increase in faecal IgA levels to those comparable of WT mice. IgA isotype class-switching has been shown to occur only within the GCs of organized gut-associated lymphoid tissues such as PPs and mLFs, and not in the diffuse lamina propria.<sup>2</sup> In another study the presence of FDC networks within gut-associated lymphoid tissues was shown to be important for the induction of intestinal IgA responses.<sup>20</sup> Taken together, our data imply that FDCs within the GCs of mLFs provide the necessary microenvironment to promote efficient interaction between luminal derived antigens and B lymphocytes to stimulate the generation of effective IgA responses.

Although PPs are absent in LT $\beta^{-/-}$  mice, the MLNs are retained,<sup>32</sup> raising the possibility that IgA isotype class-switching may also have occurred within GCs in the MLNs of WT $\rightarrow$ LT $\beta^{-/-}$  mice. However, although LT $\alpha^{-/-}$  mice lack both PPs and MLNs,<sup>33</sup> their absence in WT $\rightarrow$ LT $\alpha^{-/-}$  mice did not adversely affect faecal IgA levels, which were equivalent to those of WT mice, WT $\rightarrow$ WT mice and WT $\rightarrow$ LT $\beta^{-/-}$  mice (which do possess them). These data demonstrate that IgA isotype class-switching in response to luminal antigens can occur in the absence of MLNs, but is critically dependent upon the presence of organized lymphoid tissues such as PPs and mLFs within the intestine.

One study has suggested that ILFs are not essential for the induction of intestinal IgA responses.<sup>20</sup> In this study, mice which lacked PPs and MLNs, as a result of combined *in utero* TNFR1-signalling and LT $\beta$ R-signalling blockade, failed to induce an IgA response after oral immunization. However, TNFR1-stimulation is essential for mILF formation but not iILF formation.<sup>13</sup> Unfortunately, in the above study<sup>20</sup> ILF maturation status in TNFR1- and LT $\beta$ R-treated mice was not recorded, but it is plausible that ILF maturation was significantly impaired in these mice.

Consistent with other studies,<sup>13,14</sup> we found mLFs to be highly infrequent in the intestines of unmanipulated immunocompetent control mice in our SPF colony. However, in mice that lacked PPs, stimuli from LT-expressing, BM-derived cells significantly expanded the numbers of mLFs. The stimuli that trigger ILF maturation and the role these structures play in intestinal immune responses are not fully understood. In the absence of immunoglobulin-isotype class switching and impaired IgA production in activation-induced cytidine deaminase (AID)-deficient mice, the number of ILFs in the intestine is increased.<sup>34</sup> This implies that inflammatory responses precipitated by a relative deficiency in intestinal IgA stimulate the formation of mLFs, providing a mechanism to compensate for the absence of faecal IgA production. Indeed, our data show that ILF maturation in mice that lack PPs coincides with the restoration of faecal IgA levels to be comparable with those of immunocompetent mice. ILF formation has

also been shown to be driven by the presence of luminal bacterial flora.<sup>13,34</sup> Furthermore, following infection of mice with the parasitic helminth *Trichuris muris*,<sup>6</sup> a significant increase in the numbers of ILFs in the large intestine has also been shown to occur.<sup>6</sup>

In summary, our study has demonstrated that PP-deficiency intensifies ILF maturation in the murine small intestine. We found that mLFs, unlike iILFs, possessed large FDC networks. The area occupied by the FDCs within the mLFs of mice lacking PPs was substantial, and was equivalent to the total area occupied by FDCs in all the PPs and the few mLFs in immunocompetent mice. ILF maturation coincided with a restoration of faecal IgA to levels comparable with those in immunocompetent mice, suggesting that mLFs are important inductive sites for intestinal immune responses. Taken together, these data imply that the inducible nature of ILFs and the components within them (e.g. GCs and FDC networks) provide the intestine with an additional capacity to respond to mucosal challenges.

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# Role of the GALT in Scrapie Agent Neuroinvasion from the Intestine<sup>1</sup>

Bridget R. Glaysher and Neil A. Mabbott<sup>2</sup>

Following oral exposure, some transmissible spongiform encephalopathy (TSE) agents accumulate first upon follicular dendritic cells (FDCs) in the GALT. Studies in mice have shown that this accumulation is obligatory for the efficient delivery of the TSE agent to the brain. However, which GALTs are crucial for disease pathogenesis is uncertain. Mice deficient in specific GALT components were used here to determine their separate involvement in scrapie agent neuroinvasion from the intestine. In the combined absence of the GALTs and FDCs (lymphotoxin (LT) $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice), scrapie agent transmission was blocked. When FDC maturation was induced in remaining lymphoid tissues, mice that lacked both Peyer's patches (PPs) and mesenteric lymph nodes (wild-type (WT) $\rightarrow$ LT $\alpha^{-/-}$  mice) or PPs alone (WT $\rightarrow$ LT $\beta^{-/-}$  mice) remained refractory to disease, demonstrating an important role for the PPs. Although early scrapie agent accumulation also occurs within the mesenteric lymph nodes, their presence in WT $\rightarrow$ LT $\beta^{-/-}$  mice did not restore disease susceptibility. We have also shown that isolated lymphoid follicles (ILFs) are important novel sites of TSE agent accumulation in the intestine. Mice that lacked PPs but contained numerous FDC-containing mature ILFs succumbed to scrapie at similar times to control mice. Because the formation and maturation status of ILFs is inducible and influenced by the gut flora, our data suggest that such factors could dramatically affect susceptibility to orally acquired TSE agents. In conclusion, these data demonstrate that following oral exposure TSE agent accumulation upon FDCs within lymphoid tissue within the intestine itself is critically required for efficient neuroinvasion. *The Journal of Immunology*, 2007, 178: 3757–3766.

**T**ransmissible spongiform encephalopathies (TSEs),<sup>3</sup> or "prion diseases," are subacute neurodegenerative diseases that affect humans and both domestic and free-ranging animals. The neuropathological features within the CNS characteristically include spongiform pathology, neuronal loss, glial activation, and amyloid aggregations of an abnormally folded host protein. There is little evidence of pathology in other tissues. The host prion protein (PrP<sup>c</sup>) is widely expressed in both humans and animals, and its expression is crucial for TSE disease susceptibility (1). In TSE-affected tissues, the PrP accumulates as an abnormal, detergent-insoluble, relatively proteinase-resistant isoform, termed PrP<sup>Sc</sup>. TSE agent infectivity copurifies with PrP<sup>Sc</sup> (2) and is considered to be a major or possibly sole component of the infectious TSE agent. Indeed, the prion hypothesis argues that PrP<sup>Sc</sup> is itself the infectious agent and facilitates conversion of cellular PrP<sup>c</sup> to PrP<sup>Sc</sup>.

TSE diseases typically have long incubation periods before the onset of clinical signs, which in humans can vary from 1.5 to at least 40 years. Following peripheral exposure, many of the acquired TSE agents accumulate in lymphoid tissues such as the spleen, lymph nodes, tonsils, appendix, and Peyer's patches (PPs), before spreading to the CNS, a process termed neuroinvasion. Examples include sheep with natural scrapie (3), mule deer with chronic wasting disease (4), and humans with variant Creutzfeldt-Jakob disease (vCJD) (5). Within the lymphoid tissues of both experimentally and naturally affected hosts, early PrP<sup>Sc</sup> accumulation occurs within the germinal centers upon follicular dendritic cells (FDCs) and within tingible body macrophages (4–9). FDCs are a distinct lineage from migratory bone marrow-derived dendritic cells because they are considered to derive from stromal precursor cells, are nonphagocytic, and are nonmigratory (10). In mouse scrapie models, mature FDCs are critical for scrapie agent accumulation in lymphoid tissues, and in their absence, neuroinvasion is significantly impaired (11–14). From the lymphoid tissues, translocation to the CNS occurs via the peripheral nervous system (15, 16).

The suggestion that consumption of bovine spongiform encephalopathy-contaminated meat products is the most likely cause of vCJD in humans (17) has focused attention on the gastrointestinal tract as an important portal of TSE entry. Following intragastric or oral inoculation of rodents with scrapie, infectivity and PrP<sup>Sc</sup> accumulate first in PPs, mesenteric lymph nodes (MLNs), and ganglia of the enteric nervous system long before their detection in the CNS (18, 19). Natural sheep scrapie may also be acquired orally as PrP<sup>Sc</sup> is detected in the GALT before detection within the CNS (6). Likewise, PrP<sup>Sc</sup> is first detected in the GALT following experimental oral inoculation of mule deer fawns (*Odocoileus hemionus*) with chronic wasting disease (4). The detection of disease-specific PrP within the GALT of a human vCJD patient 8 mo before the onset of clinical signs is also consistent with the transmission of this disease by the oral route (5).

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<sup>3</sup> Abbreviations used in this paper: TSE, transmissible spongiform encephalopathy; FDC, follicular dendritic cell; GFAP, glial fibrillary acid protein; ILF, isolated lymphoid follicle; iILF, immature ILF; LT, lymphotoxin; LYVE-1, lymphatic vessel endothelial hyaluronin acid receptor-1; mILF, mature ILF; MLN, mesenteric lymph node; pAb, polyclonal Ab; PP, Peyer's patch; PrP, prion protein; vCJD, variant Creutzfeldt-Jakob disease; WT, wild type.

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The GALT comprises chiefly of appendix, tonsils, PPs, MLNs, and ILFs, but which if any of these tissues are crucial for the oral pathogenesis of TSEs is unclear. Previous studies have used a variety of immunodeficient mouse lines to study the involvement of FDCs and PPs in oral scrapie pathogenesis, including  $\text{TNF-}\alpha^{-/-}$   $\times$  lymphotoxin (LT) $\alpha^{-/-}$  mice, *aly/aly* mice,  $\beta 7^{-/-}$  mice,  $\text{RAG-1}^{-/-}$  mice, and  $\mu\text{MT}^{-/-}$  mice (20–22). However, the individual contributions of the deficiencies in lymphoid tissue, lymphoid tissue microarchitecture, or FDC development were not distinguished in the same mouse strains. In the current study, mouse models were created that lacked specific GALT components (PPs, MLNs, and ILFs) but had mature FDC networks in the remaining lymphoid tissues. The presence of FDCs in the remaining lymphoid tissues ensured that TSE agent accumulation could occur within these tissues if the missing tissues were not critical to disease pathogenesis. Using these mouse models, experiments were designed to address the following questions: first, whether TSE susceptibility is reduced in the absence lymphoid tissue within the intestine itself (e.g., PPs)? Second, whether neuroinvasion occurs from multiple sites such that the lack of one compartment (e.g., PPs) can be compensated for by the presence of another (e.g., MLNs)? Third, whether neuroninvasion can occur via other lymphoid tissues (e.g., spleen) in the absence of the GALT?

## Materials and Methods

### Mice

$\text{LT}\alpha^{-/-}$  mice (23) and  $\text{LT}\beta^{-/-}$  mice (24) were obtained from B&K Universal and were maintained on a C57BL/6 background. Age- and sex-matched C57BL/6 mice were used as immunocompetent wild-type (WT) controls in studies using  $\text{LT}\alpha^{-/-}$  mice and  $\text{LT}\beta^{-/-}$  mice.

### Gamma irradiation and bone marrow reconstitution

Bone marrow from the femurs and tibias of immunocompetent C57BL/6 WT mice was prepared as a single-cell suspension ( $3 \times 10^7$ – $4 \times 10^7$  viable cells/ml) in HBSS (Invitrogen Life Technologies). Recipient adult (6–8 wk old)  $\text{LT}\alpha^{-/-}$  mice,  $\text{LT}\beta^{-/-}$  mice, and C57BL/6 mice were gamma irradiated (950 rad) and 24 h later reconstituted with 0.1 ml of bone marrow by injection into the tail vein.

### PP-deficient mice

To create progeny mice deficient in PPs, timed pregnant C57BL/Dk mice were given a single i.v. injection of 100  $\mu\text{g}$  of a fusion protein containing the soluble  $\text{LT}\beta\text{R}$  domain linked to the Fc portion of human IgG1 ( $\text{LT}\beta\text{R}$ -Ig; Ref 25) on day 11.5 of gestation (26).

### Scrapie agent inoculation

For oral inoculation mice were fed individual food pellets doused with 50  $\mu\text{l}$  of a 1.0% (w/v) scrapie brain homogenate prepared from mice terminally affected with ME7 scrapie. Where indicated, separate groups of mice were inoculated by intracranial injection with 20  $\mu\text{l}$  of a 1.0% (w/v) scrapie mouse brain homogenate (containing  $\sim 1 \times 10^{4.5}$  ID<sub>50</sub> units). Following challenge, animals were coded and assessed weekly for signs of clinical disease and killed at a standard clinical end point (27). Scrapie diagnosis was confirmed by histopathological assessment of vacuolation in the brain. Where indicated, some mice were sacrificed 70 days postchallenge, and small intestines, MLNs, and spleens taken for further analysis. For the construction of lesion profiles, vacuolar changes were scored in nine gray-matter areas of brain as described previously (28).

### Immunohistochemical and immunofluorescent analysis

Spleens and MLNs were removed and snap-frozen at the temperature of liquid nitrogen. Small intestines from each mouse were divided into three roughly equal parts, gently squeezed to remove gut contents, coiled, embedded in Tissue-Tek OCT Compound (Bayer), and snap-frozen at the temperature of liquid nitrogen. Serial frozen sections (10  $\mu\text{m}$  in thickness) were cut on a cryostat. FDCs were visualized by staining with mAb 8C12 to detect CR1 (CD35) or mAb 7G6 to detect CR2/CR1 (CD21/CD35; BD Biosciences Pharmingen). Complement components C3 and C4 were de-

tected using mAb RMC7H8 (Connex) and mAb FDC-M2 (AMS Biotechnology), respectively. Cellular PrP<sup>c</sup> was detected using PrP-specific polyclonal Ab (pAb) 1B3 (29). B lymphocytes were detected using mAb B220 to detect CD45R (Caltag Laboratories). M cells were detected using biotin-conjugated *Ulex europaeus* agglutinin I (UEA-I; Vector Laboratories). Lymphatic vessels were detected using lymphatic vessel endothelial hyaluronic acid receptor (LYVE-1)-specific antiserum (Upstate Biotechnology). Nerve fibers and supportive cells were detected using S100-specific antiserum (DakoCytomation).

For the detection of disease-specific PrP (PrP<sup>d</sup>) in brain tissue and small intestines, tissues were fixed in periodate-lysine-paraformaldehyde and embedded in paraffin wax. Sections (thickness, 6  $\mu\text{m}$ ) were deparaffinized and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121°C, hydration), and subsequent immersion in formic acid (98%) for 5 min (7). This pretreatment enhances the detection of PrP<sup>d</sup>. Sections were then stained with the PrP-specific pAb 1B3 (29). Glial fibrillary acid protein (GFAP) was detected on adjacent brain sections using rabbit GFAP-specific antiserum (DakoCytomation). To detect FDCs and B lymphocytes in paraffin-embedded small intestines, sections were deparaffinized and pretreated with Target Retrieval Solution (DakoCytomation) and immunostained with mAb 7G6 and mAb B220, respectively, as described above.

For light microscopy, following the addition of primary Abs, biotin-conjugated species-specific secondary Abs (Strattech) were applied followed by alkaline phosphatase or HRP coupled to the avidin/biotin complex (Vector Laboratories). Vector Red (Vector Laboratories) and diaminobenzidine were used as substrates, respectively, and sections were counterstained with hematoxylin to distinguish cell nuclei. For fluorescent microscopy, following the addition of primary Ab, species-specific secondary Abs coupled to Alexa Fluor 488 (green) or Alexa Fluor 594 (red) dyes (Invitrogen Life Technologies) were used. Sections were mounted in fluorescent mounting medium (DakoCytomation) and examined using a Zeiss LSM5 confocal microscope (Zeiss).

### ILF enumeration and analysis

Entire small intestines from each mouse were divided into three equal parts, coiled, fixed in paraformaldehyde, and embedded in paraffin wax. Serial sections (10  $\mu\text{m}$  in thickness) were deparaffinized and immunostained with antisera specific for CD45R or CR2/CR1 as described above and examined using a confocal microscope. The total number of B lymphocyte-containing ILFs in the entire small intestine of each mouse analyzed was counted microscopically using CD45R expression for identification. The maturity of each ILF was determined according to size and the location and density of component cells as described previously (30). The presence or absence of FDCs (CR2/CR1<sup>+</sup>CD45R<sup>+</sup> cells) within each ILF was recorded.

### Immunoblot detection of PrP<sup>Sc</sup>

Spleen fragments (~20 mg) or MLNs (approximately half the total from each mouse assayed) were prepared as 10% (w/v) tissue homogenates, and PrP<sup>Sc</sup> was enriched by sodium phosphotungstic acid precipitation (31) and treated in the presence or absence of proteinase K (40  $\mu\text{g}/\text{ml}$ , 60 min, 37°C; VWR). Following enrichment, pellets were resuspended and diluted to 0.5 mg protein/ml, and 10  $\mu\text{l}$  was electrophoresed through SDS-PAGE gels (12%) polyacrylamide gels (Invitrogen Life Technologies). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) by semidry blotting. PrP was detected with the PrP-specific mouse mAb 8H4 (32), followed by HRP-conjugated goat anti-mouse antiserum. Bound HRP activity was detected with Supersignal West Dura Extended Duration Substrate (Pierce).

### Statistical analysis

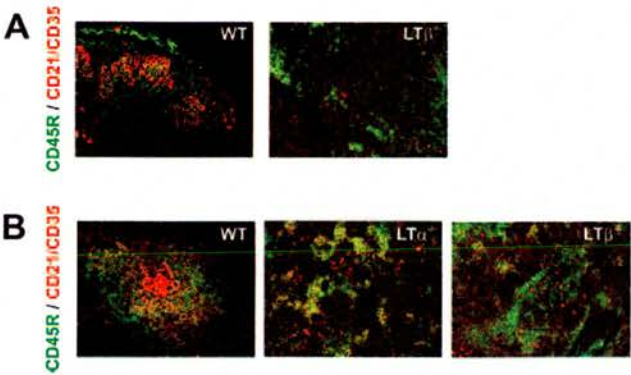
Data are presented as mean  $\pm$  SE. Significant differences between samples in different groups were sought by one-way ANOVA. Values of  $p < 0.05$  were accepted as significant.

## Results

### Effect of lymphotoxin deficiency on scrapie accumulation in lymphoid tissues

The intestines of  $\text{LT}\alpha^{-/-}$  mice and  $\text{LT}\beta^{-/-}$  mice lack PPs as  $\text{LT}\beta\text{R}$  signaling during embryonic development is required for their formation (30, 33, 34). In addition  $\text{LT}\alpha^{-/-}$  mice also lack the MLNs as  $\text{LT}\alpha$  deficiency blocks their formation (34). However,





**FIGURE 1.** Immunohistochemical detection of FDCs within the MLNs (A) and spleens (B) of WT mice,  $LT\alpha^{-/-}$  mice, and  $LT\beta^{-/-}$  mice. CD21/CD35-expressing (red) FDCs and organized B lymphocyte follicles (CD45R-positive cells; green) were detected in the tissues of WT mice. No FDCs were detected in tissues from  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice. Original magnification:  $\times 200$ .

postnatal stimulation via lymphocyte-derived  $LT\alpha_1\beta_2$  is critically required to maintain FDCs in their differentiated state (35). As a consequence, the remaining MLNs and spleens of  $LT\beta^{-/-}$  mice (Fig. 1, A and B, respectively) and the spleens of  $LT\alpha^{-/-}$  mice (Fig. 1B) are deficient in FDCs. Because FDCs are critical for the efficient neuroinvasion of the ME7 scrapie agent from the intestine (13), we first investigated scrapie pathogenesis in the combined absence of GALT (MLNs and/or PPs) and FDCs in the remaining lymphoid tissues in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice.

In this study, the normal cellular form of the PrP is referred to as  $PrP^c$ , and two terms ( $PrP^{Sc}$  or  $PrP^d$ ) are used to describe the disease-specific, abnormal accumulations of PrP that are characteristically found only in TSE-affected tissues and considered to represent the presence of the TSE agent (2). Disease-specific PrP accumulations are relatively resistant to proteinase digestion, whereas cellular  $PrP^c$  is destroyed by this treatment. Where we were able to confirm this resistance by proteinase K treatment of samples and analysis by immunoblot,  $PrP^{Sc}$  is used as a biochemical marker for the presence of the TSE agent. Unfortunately, treatment of tissue sections with proteinase K destroys the microarchitecture. Therefore, tissue sections were fixed and pretreated to enhance the detection of the disease-specific abnormal accumula-

tions of PrP ( $PrP^d$ ), whereas cellular  $PrP^c$  is denatured by these treatments (7). We have shown in a series of studies that these  $PrP^d$  accumulations occur only in the tissues of TSE-affected animals and correlate closely with the presence of the TSE agent (7, 8, 11, 13, 19). Expression levels of cellular  $PrP^c$  in tissues from uninfected mice were analyzed on acetone-fixed frozen sections. Immunocompetent C57BL/6 WT mice,  $LT\alpha^{-/-}$  mice, and  $LT\beta^{-/-}$  mice were inoculated orally with the ME7 scrapie strain. Within 70 days of oral inoculation of WT mice with the ME7 scrapie agent, strong accumulations of  $PrP^d$  and agent infectivity are found within PPs (Ref. 13; N. A. Mabbott, unpublished observations). High levels of proteinase K-resistant  $PrP^{Sc}$  and agent infectivity are also found within MLNs (Fig. 2A, lanes 2 and 4; Ref. 13; N. A. Mabbott, unpublished observations) and are sustained until the terminal stage of disease (Fig. 2C, lanes 2 and 4). However, in the MLNs of  $LT\beta^{-/-}$  mice or the mesenteric membranes taken from MLN-deficient  $LT\alpha^{-/-}$  mice,  $PrP^{Sc}$  was undetectable both 70 days (Fig. 2A) and 545 days (Fig. 2C) after inoculation. No  $PrP^{Sc}$  was detected in any of the spleens from WT mice,  $LT\alpha^{-/-}$  mice, and  $LT\beta^{-/-}$  mice assayed 70 days after inoculation (Fig. 2B). However, strong accumulations of  $PrP^{Sc}$  were found in the spleens of WT mice taken at the terminal stage of disease (Fig. 2D), but the subsequent accumulation in the spleens of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice was blocked (Fig. 2D). Thus, in the absence of PPs and FDCs in remaining lymphoid tissues,  $PrP^{Sc}$  accumulation in MLNs and the spleen is blocked.

Effect of lymphotoxin deficiency on scrapie susceptibility

We next determined the effect of the combined GALT and FDC deficiency in  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice on scrapie susceptibility. All orally inoculated WT mice succumbed to clinical TSE disease with a mean incubation period of  $311 \pm 3$  days ( $n = 20$ ; Table I). In contrast, LT deficiency dramatically affected scrapie-susceptibility as all  $LT\alpha^{-/-}$  mice ( $n = 13$ ) and  $LT\beta^{-/-}$  mice ( $n = 18$ ) remained free of the signs of scrapie up to at least 545 days after oral inoculation (Table I). Characteristic  $PrP^d$  accumulation, reactive astrocytes, and spongiform pathology were detected in the brains of all clinically affected WT mice (Fig. 3A). The severity and distribution of the pathological vacuolation within the brains of WT mice was typical for oral infection with the ME7 scrapie strain (Fig. 3B). However, none of the pathological

**FIGURE 2.**  $PrP^{Sc}$  accumulation in the MLNs and spleens of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice is blocked following oral inoculation with scrapie. A and C, MLNs (or the mesenteric membrane containing the region where MLNs would have been in  $LT\alpha^{-/-}$  mice) and spleens (B and D) were collected 70 days after oral inoculation with the scrapie agent (A and B) or at the terminal stage of disease (C and D). Samples were treated in the presence (+) or absence (–) of proteinase K (PK) before electrophoresis. After PK treatment, a typical three-band pattern was observed between molecular mass values of 20–30 kDa, representing unglycosylated, monoglycosylated, and diglycosylated isomers of PrP (in order of increasing molecular mass). dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie.

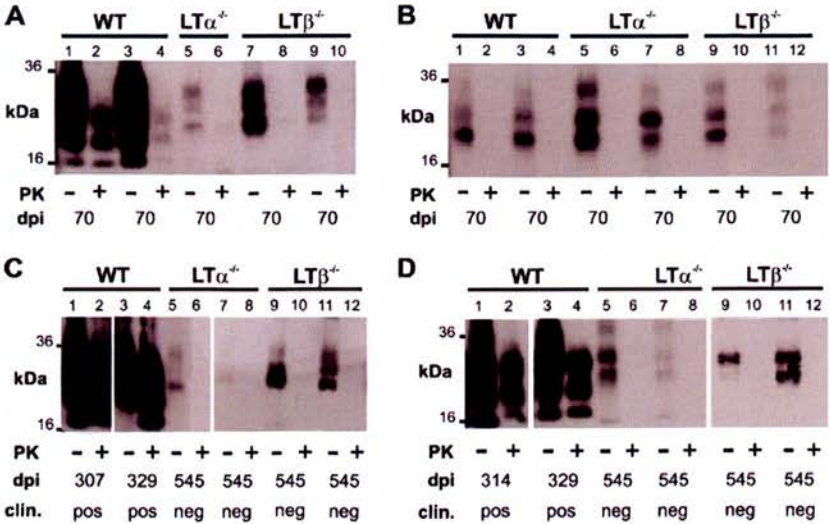




Table 1. Effect of lymphotoxin deficiency on scrapie agent susceptibility

Mouse Strain	Presence at Time of Inoculation					Oral Inoculation		Intracranial Inoculation	
	PPs	mILFs <sup>a</sup>	MLNs	SPL	FDCs	Incidence <sup>b</sup>	Mean incubation period (days) ± SE	Incidence	Mean incubation period (days) ± SE
WT	+ <sup>c</sup>	5	+	+	+	20/20	311 ± 3	6/6	173 ± 4
LTα <sup>-/-</sup>	—	0	—	+	—	0/13	13X > 545	5/5	175 ± 4
LTβ <sup>-/-</sup>	—	0	+	+	—	0/18	18X > 545	6/6	172 ± 3

<sup>a</sup> Number of mILFs present in the small intestine at the time of inoculation.

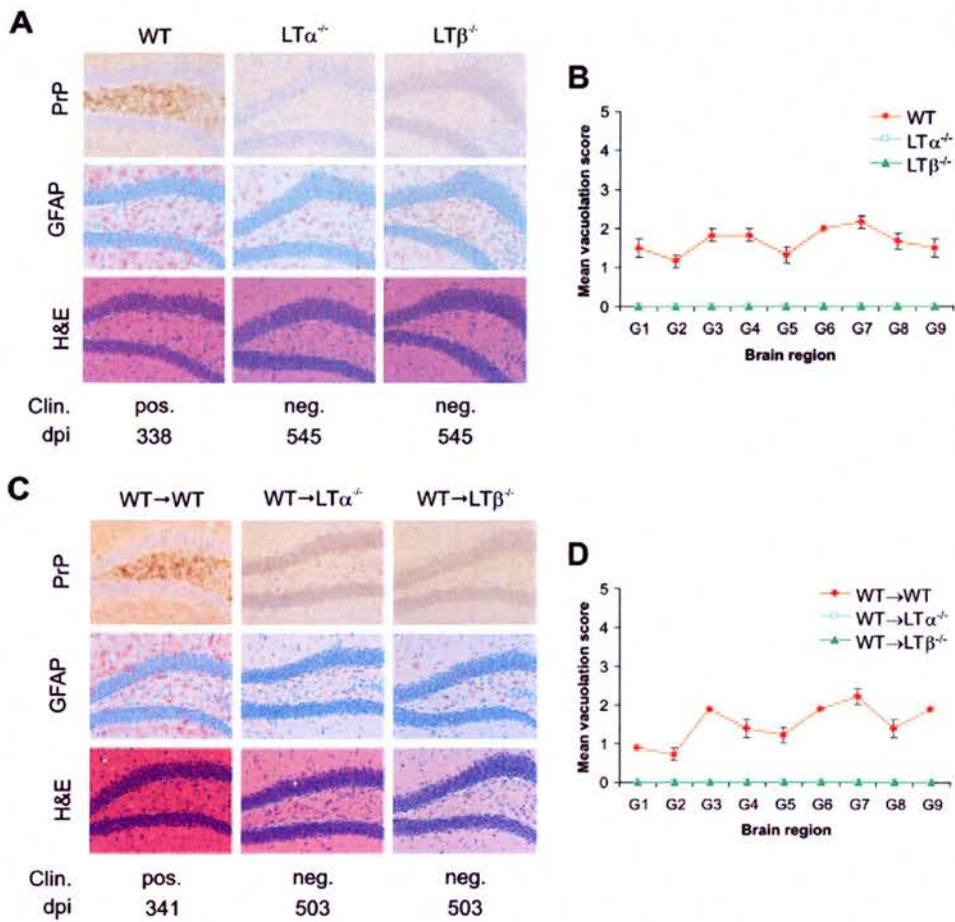
<sup>b</sup> Incidence = number of animals affected/number of animals tested. The notation “NX > 545” means that mice were free of the clinical and pathological signs of scrapie up to at least this time after inoculation.

<sup>c</sup> +, present; —, absent.

characteristics of TSE disease were detected within the brains of any of the surviving LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice (Fig. 3, A and B).

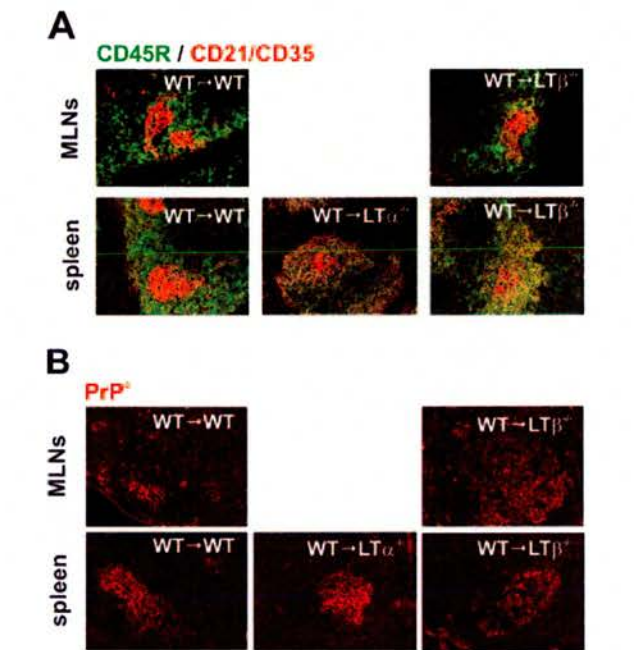
When inoculated with the scrapie agent directly into the CNS (by intracranial injection) WT mice, LTα<sup>-/-</sup> mice, and LTβ<sup>-/-</sup>

mice developed clinical scrapie with similar incubation periods of 172–175 days (Table 1). These data confirm that the apparent resistance of LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice to orally inoculated scrapie agent could not be attributed to a role for LTβR signaling in the development of pathology within the CNS.



**FIGURE 3.** Immunohistochemical and pathological analysis of brain tissue from terminally scrapie-affected mice and mice that remained free of the signs of disease at the end of the experiment. *A* and *B*, Brains from WT mice, LTα<sup>-/-</sup> mice, and LTβ<sup>-/-</sup> mice inoculated orally with the scrapie agent. *C* and *D*, Brains from WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice inoculated orally with the scrapie agent. Large PrP<sup>Sc</sup> accumulations (brown; upper rows), reactive astrocytes expressing high levels of GFAP (red; middle rows), and spongiform pathology (lower rows) were detected in the hippocampi of all clinically scrapie-affected WT mice (*A*) and WT→WT mice (*C*), but not in the brains clinically negative LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice (*A*), and WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice (*C*). All sections were counterstained with hematoxylin (blue). Original magnification: ×200. dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie. *B* and *D*, Pathological assessment of the spongiform change (vacuolation) in brains from terminally scrapie-affected mice and mice that remained free of the signs of disease at the end of the experiment. Vacuolation was scored on a scale of 0–5 in the following gray matter (G1–G9) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex. Each point represents mean vacuolation score ± SE for groups of 6–20 mice.





**FIGURE 4.** Immunohistochemical analysis of the microarchitecture of the MLNs and spleen at the time of scrapie challenge. *A*, CD21/CD35-expressing (red) FDCs and organized B lymphocyte follicles (CD45R-positive cells; green) were detected in the MLNs of WT→WT mice and WT→LTβ<sup>-/-</sup> mice (upper row) and spleens of WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice (lower row). *B*, The levels of PrP<sup>Sc</sup> expressed upon FDCs (red) were similar in the MLNs of WT→WT mice and WT→LTβ<sup>-/-</sup> mice (upper row) and spleens of WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice (lower row). Original magnification: ×200.

*Reconstitution of LT-deficient mice with WT bone marrow induces FDC development in remaining lymphoid tissues but not the development of missing lymphoid tissues*

Next, the effects of GALT deficiency, but not FDC deficiency, on scrapie pathogenesis were investigated. Adult WT mice, LTα<sup>-/-</sup> mice, and LTβ<sup>-/-</sup> mice were reconstituted with LT-expressing WT bone marrow (termed WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice, respectively). Within 35 days of WT bone marrow transfer, FDC networks were induced in the remaining lymphoid tissues of LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice (Fig. 4*A*), but such treatment did not induce the development of missing lymphoid tissues (Table II). Expression of the cellular form of the PrP,

PrP<sup>C</sup>, upon FDCs within the MLNs (where present) and spleens of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice appeared similar to that in tissues from WT→WT mice (Fig. 4*B*).

*Scrapie pathogenesis in the absence of MLNs and/or PPs*

Groups of WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice were inoculated orally with scrapie 35 days after reconstitution with WT bone marrow. Strong accumulations of PrP<sup>d</sup> were observed within the PPs of WT→WT mice within 70 days of inoculation (Fig. 5*A*) and were maintained until the terminal stage of disease (Fig. 5*B*). The distribution of the PrP<sup>d</sup> within PPs was consistent with accumulation upon FDCs (8, 13). However, PrP<sup>d</sup> accumulation within the intestines of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice was blocked (Fig. 5).

In the MLNs, PrP<sup>Sc</sup> was undetectable in tissues from WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice assayed 70 days after inoculation (Fig. 6*A*), although PrP<sup>Sc</sup> was detected in one of the spleens from a WT→WT mice taken at this time (Fig. 6*B*, lane 2). At the terminal stage of disease strong accumulations of PrP<sup>Sc</sup> were found in the MLNs and spleens of WT→WT mice (Fig. 6, *C* and *D*). However, the accumulation of PrP<sup>Sc</sup> in the MLNs (where present) and spleens of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice remained undetectable (Fig. 6, *C* and *D*).

We next determined the effect of MLN and/or PP deficiency on disease susceptibility. All orally inoculated WT→WT mice succumbed to clinical TSE disease with a mean incubation period of 337 ± 4 days (*n* = 8, Table II). In contrast, in the absence of both PPs and MLNs, scrapie susceptibility was dramatically affected as all orally inoculated WT→LTα<sup>-/-</sup> mice (*n* = 8) remained free of the signs of scrapie up to at least 503 days after inoculation (Table II). Furthermore, in the absence of PPs alone, scrapie susceptibility was also dramatically affected as all WT→LTβ<sup>-/-</sup> mice (*n* = 7) remained free of clinical scrapie (Table II). Typical neuropathological characteristics of TSE infection were detected within the brains of all WT→WT animals that developed clinical signs of scrapie (Fig. 3, *C* and *D*). None of these characteristics were detected within the brains of any of the surviving WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice (Fig. 3, *C* and *D*).

In this study, a highly sensitive immunoblot method was used to detect PrP<sup>Sc</sup> in our tissue samples (31). Therefore, we are confident that in this study the combined absence of 1) PrP<sup>Sc</sup> in the GALT and spleen, 2) PrP<sup>d</sup> and neuropathological signs within the CNS, and 3) clinical signs of TSE disease, is convincing evidence that transmission was blocked in scrapie-inoculated LTα<sup>-/-</sup> mice, LTβ<sup>-/-</sup> mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice.

**Table II.** *Reconstitution of LT-deficient mice with WT bone marrow induces FDC maturation in remaining lymphoid tissues but does not restore susceptibility to orally inoculated scrapie agent*

Mouse Strain <sup>a</sup>	Presence at Time of Inoculation					Oral Scrapie Inoculation	
	PPs	mILFs <sup>b</sup>	MLNs	SPL	FDCs	Incidence	Mean incubation period (days) ± SE
WT→WT	+ <sup>c</sup>	0	+	+	+	8/8	337 ± 4
WT→LTα <sup>-/-</sup>	—	0	—	+	+	0/8	5X > 503 <sup>d</sup>
WT→LTβ <sup>-/-</sup>	—	0	+	+	+	0/7	4X > 503 <sup>e</sup>

<sup>a</sup> Mice were inoculated orally with the scrapie agent 35 days after gamma irradiation and reconstitution with WT bone marrow.

<sup>b</sup> Number of mILFs present in the small intestine at the time of inoculation.

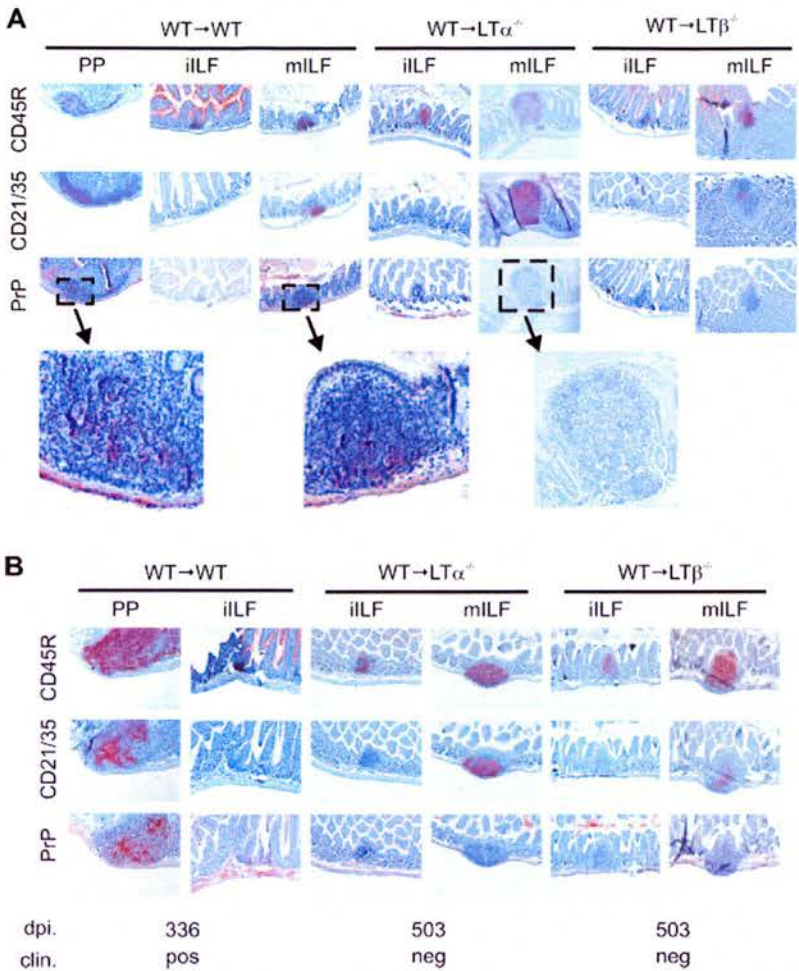
<sup>c</sup> +, present; —, absent.

<sup>d</sup> Three mice were killed 406, 450, and 467 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull. No histopathological signs of scrapie were detected in the brain (data not shown).

<sup>e</sup> Three mice were killed 413, 455, and 455 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull. No histopathological signs of scrapie were detected in the brain (data not shown).



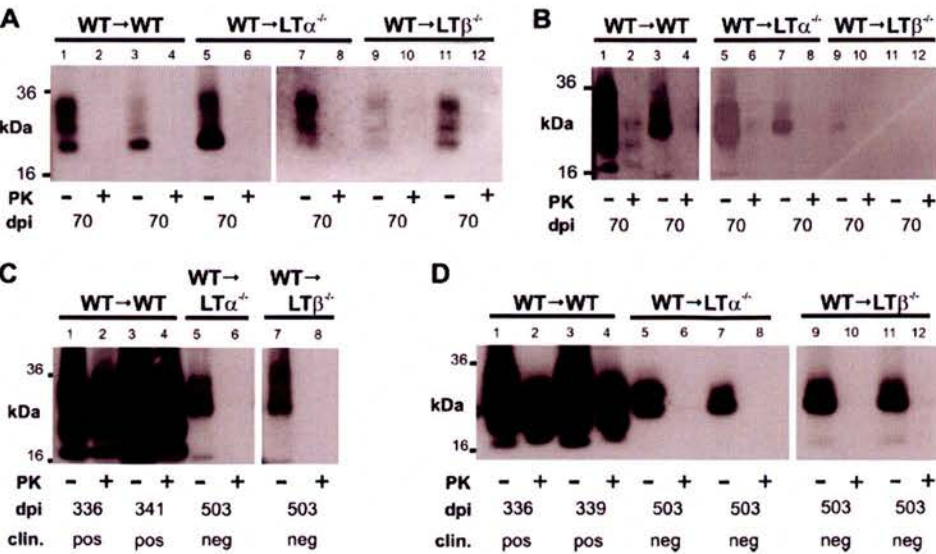
**FIGURE 5.** Detection of PrP<sup>d</sup> in the intestines of WT→WT mice, WT→LTα<sup>-/-</sup> mice, and WT→LTβ<sup>-/-</sup> mice following oral inoculation with the scrapie agent. Small intestines were collected (A) 70 days after oral inoculation or (B) at the terminal stage of disease. Adjacent sections were stained with CD45R-specific antiserum B220 to detect B lymphocytes (red), CD21/35-specific antiserum to detect CR2/CR1-expressing FDCs (red), and PrP-specific antiserum 1B3 (red). Strong PrP<sup>d</sup> staining colocalized with CD21/35 expressing FDCs in the PPs and the few mLIFs of WT→WT mice 70 days after oral inoculation (A) and at the terminal stage of disease (B). PrP<sup>d</sup> was not detected in the mLIFs of scrapie-inoculated WT→LTα<sup>-/-</sup> mice or WT→LTβ<sup>-/-</sup> mice. dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie. Original magnification: ×100.



*Mature ILFs contain FDCs*

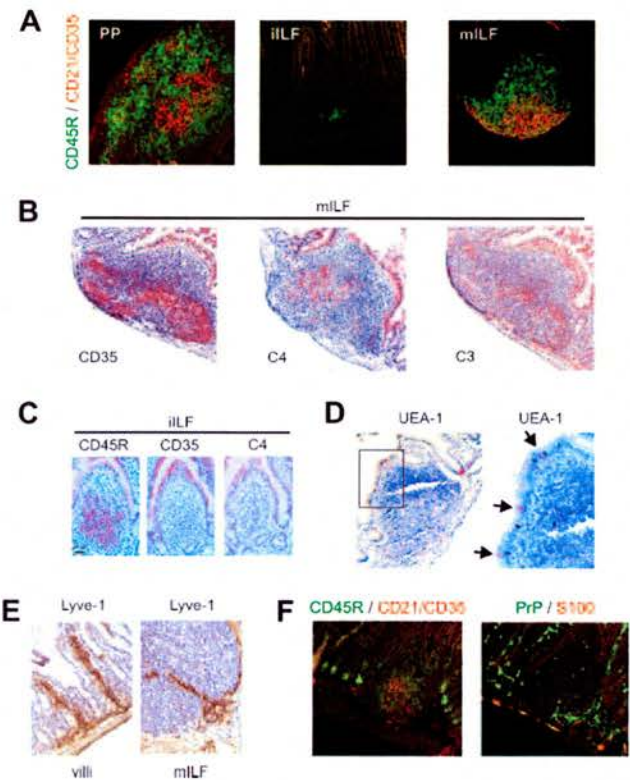
Novel lymphoid clusters termed ILFs have been identified along the antimesenteric wall of the mucosa of the intestine (36). The maturity of ILFs has previously been defined according size and the location and density of component cells (30). Whereas immature ILFs (iILFs) comprise loosely clustered CD45R<sup>+</sup> (B220) cells located at the base of villi (Fig. 7A), mature ILFs (mILFs) are

organized nodular structures, of width greater than one villus and comprise a single B lymphocyte follicle (Fig. 7A) with an overlying M cell-containing epithelium similar to the follicle associated epithelium of PPs (Fig. 7D). Using these criteria, double immunostaining for B lymphocytes (CD45R<sup>+</sup> cells) and FDCs (CR2/CR1<sup>+</sup>CD45R<sup>-</sup> cells) on the same tissue section revealed that the presence of FDCs could also be reliably used to distinguish ILF



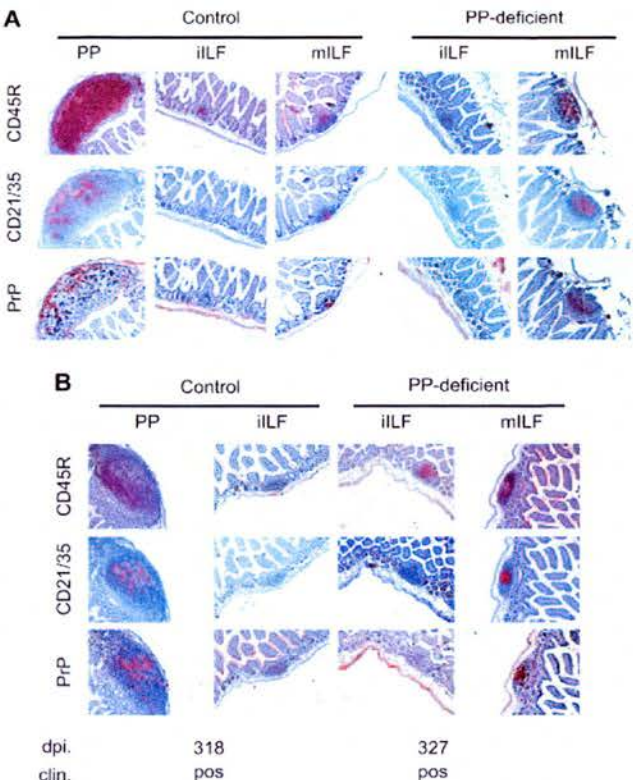
**FIGURE 6.** PrP<sup>Sc</sup> accumulation in the MLNs and spleens of WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice is blocked following oral inoculation with the scrapie agent. A and C, MLNs (or the mesenteric membrane containing the region where MLNs would have been in WT→LTα<sup>-/-</sup> mice) and spleens (B and D) were collected 70 days after oral inoculation with the scrapie agent (A and B) or at the terminal stage of disease (C and D). Samples were treated in the presence (+) or absence (-) of proteinase K (PK) before electrophoresis. dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie.





**FIGURE 7.** Immunohistochemical characterization of mILFs. *A*, iILFs typically comprise a loose aggregation of B lymphocytes at the base of a villus (CD45R-positive cells; green), whereas mILFs contain both B lymphocytes and CR2/CR1-expressing FDCs (CD21/CD35-positive cells; red). *B*, Complement components C4 (red; middle) and C3 (red; right) in association with CR1-expressing FDCs (CD35-positive cells; red; left) were detected within mILFs indicating the presence of functional FDCs. *C* In iILFs, CR1-expressing FDCs (CD35-positive cells; red; middle) and associated C4 (red; right) are absent. *D*, M cells (UEA-1-positive cells; red; arrowheads) were detected within the epithelium overlying mILFs. *Right panel* shows an enlargement of the boxed area. *E*, Lymphatic vessels (LYVE-1-expressing cells; brown) were detected within the cores of villi (*left panel*) and within and around mILFs (*right panel*). *F*, PrP (green) and S-100 (red)-expressing nerve fibers, and supportive cells were detected within the gut wall and in close association with mILFs. In *B–E*, sections were counterstained with hematoxylin (blue). Each image is orientated such that the intestinal lumen is uppermost. Original magnification:  $\times 200$ .

maturation status. In this study,  $>1000$  ILFs were analyzed, and our data showed that all mILFs consistently possessed large networks of immune complex-trapping FDCs (Fig. 7, *A* and *B*). FDCs were never detected in iILFs (Fig. 7, *A* and *C*). Like PPs, ILFs lack afferent lymphatics, but LYVE-1-expressing cells were observed in close association with ILFs, indicating the presence of efferent lymphatics (Fig.



**FIGURE 8.** Detection of PrP<sup>d</sup> in the intestines of control mice and PP-deficient mice following oral inoculation with the scrapie agent. Small intestines were collected (*A*) 70 days after oral inoculation or (*B*) at the terminal stage of disease. Adjacent sections were stained with CD45R-specific antiserum B220 to detect B lymphocytes (red), CD21/35-specific antiserum to detect CR2/CR1-expressing FDCs (red), and PrP-specific antiserum 1B3 (red). At each time point after scrapie inoculation, strong PrP<sup>d</sup> staining was found to colocalize with CD21/35-expressing FDCs in the PPs and the mILFs of control mice and also in the mILFs of PP-deficient mice. dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie. Original magnification:  $\times 100$ .

7*E*). S-100-expressing nerve fibers and supportive cells were detected within the gut wall and in close association with mILFs (Fig. 7*F*).

*Involvement of mILFs in scrapie agent neuroinvasion from the intestine*

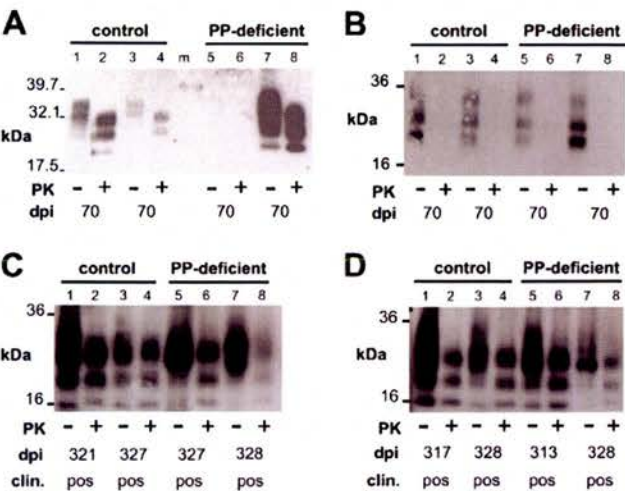
Having demonstrated that mILFs contain large FDC networks we next determined whether scrapie agent neuroinvasion from the intestine could also occur from mILFs. Stimulation via LT $\beta$ R is also important for the development of ILFs as these structures are absent in LT-deficient mice (30, 36). However, unlike PPs, ILF formation occurs postnatally, and their development in LT-deficient

Table III. Effect of PP deficiency on susceptibility to orally inoculated scrapie

Mice	Presence at Time of Inoculation					Oral Scrapie Inoculation	
	PPs	mILFs <sup>a</sup>	MLNs	SPL	FDCs	Incidence	Mean incubation period (days) $\pm$ SE
C57BL/Dk (control)	+ <sup>b</sup>	2	+	+	+	16/16	319 $\pm$ 3
PP deficient <sup>c</sup>	–	20	+	+	+	10/10	313 $\pm$ 6

<sup>a</sup> Number of mILFs present in the small intestine at the time of inoculation.  
<sup>b</sup> +, present; –, absent.  
<sup>c</sup> Mice treated in utero with LT $\beta$ R-Ig on day 11.5 of gestation to ablate PP formation.





**FIGURE 9.** PrP<sup>Sc</sup> accumulation in the MLNs and spleens of control mice and PP-deficient mice following oral inoculation with scrapie. MLNs (A and C) and spleens (B and D) were collected 70 days after oral inoculation with the scrapie agent (A and B) or at the terminal stage of disease (C and D). Samples were treated in the presence (+) or absence (–) of proteinase K (PK) before electrophoresis. Lane M contained molecular mass markers. dpi, day postinoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie.

mice can be restored by reconstitution with WT bone marrow (30, 36). In the experiments described above (Table II), no FDC-containing mILFs were present within the intestines of WT→WT mice, WT→LTα<sup>–/–</sup> mice, and WT→LTβ<sup>–/–</sup> mice at the time of scrapie inoculation, as these structures take much longer than 35 days to develop after WT bone marrow transfer (~8–12 wk; Refs. 30 and 37). The numbers of mILFs in the intestines of immunocompetent control mice in our specific pathogen-free colony are extremely low or absent (typically 0–6 mILFs/mouse). However, in the absence of PPs, LTβR stimulation substantially increases the number of mILFs in the intestine (30). We therefore created mice that lacked PPs but contained numerous mILFs in their intestines through treatment of pregnant C57BL/Dk mice with LTβR-Ig (26, 30). As expected, PP formation in the progeny mice was blocked (termed PP-deficient mice), but the development of a significantly greater number of FDC-containing mILFs was induced when compared with untreated C57BL/Dk (control) mice (*p* ≤ 0.001; Table III).

Control and PP-deficient mice (81–110 days old) were inoculated orally with the scrapie agent. Strong accumulations of PrP<sup>d</sup> were observed upon FDCs within each of the numerous mILFs of PP-deficient mice (~20 mILFs per PP-deficient mouse, *n* = 14) and the PPs and occasional mILFs in control mice within 70 days of oral inoculation (Fig. 8A) and at the terminal stage of disease (Fig. 8B). PrP<sup>d</sup> accumulations were never observed within iILFs from each mouse group.

In the MLNs, PrP<sup>Sc</sup> was detected in all tissues from control mice 70 days after inoculation, but the levels detected in tissues from PP-deficient mice were variable (Fig. 9A). However, high levels of PrP<sup>Sc</sup> levels were detected in MLNs from each mouse group at the terminal stage of disease (Fig. 9C). No PrP<sup>Sc</sup> was detected in any of the spleens taken from control mice and PP-deficient mice 70 days after inoculation (Fig. 9B), but strong accumulations were observed at the terminal stage of disease (Fig. 9D).

Additional experiments demonstrated that the susceptibility of PP-deficient mice to orally inoculated scrapie agent was not significantly affected, as all control mice and PP-deficient mice succumbed to clinical TSE disease with similar incubation periods (Table III) and dis-

played similar neuropathological characteristics within the brain (data not shown). Together, these data imply that FDCs within mILFs are also important sites of TSE agent neuroinvasion from the intestine.

Discussion

In this study, we show that in the combined absence of the GALT and FDCs at the time of inoculation, scrapie agent transmission from the gastrointestinal tract is blocked. Furthermore, when FDC maturation is induced in remaining lymphoid tissues, mice that lack PPs remain refractory to oral scrapie challenge. Although the scrapie agent also accumulates upon FDCs within the MLNs shortly after inoculation, our data show that MLNs are dispensable for TSE agent neuroinvasion from the intestine. We show that mILFs along the antimesenteric wall of the small intestine contain large FDC networks, and like PPs, are important sites of TSE agent neuroinvasion. Together, these data demonstrate that TSE agent accumulation upon FDCs within GALT in the intestine itself (e.g., PPs and mILFs) is critically required for efficient neuroinvasion following oral exposure.

When considered in the context of recent studies, our data suggest that TSE agent neuroinvasion from the intestine occurs via the following pathway. Following oral inoculation, FDCs are critical initial sites of TSE agent accumulation within the GALT (13). In the absence of FDCs at the time of inoculation, TSE agent accumulation in the PP and subsequent neuroinvasion are blocked (13). In the current study, we show that in the absence of PPs, but not FDCs within other lymphoid tissues, neuroinvasion is likewise blocked indicating a critical role for PPs and the FDCs within them in TSE agent neuroinvasion from the intestine. We also show that mILFs, like PPs, are novel and important sites of TSE agent neuroinvasion from the intestine. Because the number and maturation status of ILFs is dramatically influenced by luminal stimuli (30, 38, 39), our data suggest that the presence of mILFs will significantly affect TSE susceptibility. Although early TSE agent accumulation also occurs within the MLNs following oral inoculation, we demonstrate that their presence does not influence disease pathogenesis. Likewise, FDCs within the spleen were also unable to compensate for the absence of the PPs. Together these data imply that neuroinvasion most likely occurs directly from lymphoid tissue within the intestine following accumulation upon FDCs. This conclusion is congruent with data from an epidemiological, mathematical, and pathological study, which suggested that for sheep, cattle, and humans, there was an association between development of lymphoid tissues in the gastrointestinal tract and susceptibility to natural TSE infection (40). The precise role that FDCs play in TSE pathogenesis awaits definitive demonstration, but they appear to amplify the levels of the TSE agent above the threshold required to achieve neuroinvasion (11–14). When and how the TSE agent spreads from the FDCs within PPs to the peripheral nervous system is likewise unknown. FDC depletion 14 days after oral inoculation does not affect pathogenesis, implying that neuroinvasion occurs from a very early stage postexposure and before the agent has disseminated to other lymphoid tissues (13). We show that within mILFs, like PPs (19), FDC networks are situated in close association with the nerve fibers that run along the gut wall. As the distance between FDCs and peripheral nerves appears to modulate the rate of TSE agent neuroinvasion (16), it is likely that the close association of FDCs with the nerve fibers that run along the gut wall facilitates the rapid spread of the TSE agent from PPs and mILFs to neighboring enteric nerves. Indeed, studies have demonstrated that PrP<sup>d</sup> accumulates within enteric ganglia soon after oral inoculation (19).

How TSE agents are initially acquired from the gut lumen and cross the intestinal epithelium is not known, but several mechanisms have been implicated including transcytosis by M cells (41)



and possibly also capture and transport by migratory bone marrow-derived dendritic cells (42). In the current study, any potential effects on dendritic cell migration to the spleen, due to a lack of  $LT\alpha_1\beta_2$  expressing B lymphocytes (43), were restored by WT bone marrow reconstitution. However, our data cannot exclude the possibility that the lack of M cells within the follicle associated epithelia of  $LT\alpha^{-/-}$  mice,  $LT\beta^{-/-}$  mice,  $WT\rightarrow LT\alpha^{-/-}$  mice, and  $WT\rightarrow LT\beta^{-/-}$  mice at the time of inoculation also contributes to their resistance to orally inoculated scrapie agent.

The inability of the MLNs or spleen to compensate for the absence of the PPs is unlikely to be due to fundamental differences in the nature of the FDCs at each of these sites as no significant differences between the function of FDCs from these tissues has been described, and each population appears to express similar levels of PrP<sup>c</sup> and CR1/CR2. Following oral inoculation, the TSE agent accumulates first in PPs and is subsequently distributed via the lymph and/or blood-stream to the MLNs and spleen (Ref. 13 and current study). In the absence of the PPs, our data show that this down-stream distribution is blocked.

Diet (e.g., consumption of TSE agent-contaminated food) and PrP genotype (44, 45) are important risk factors that affect TSE susceptibility, but others remain to be determined. Recent studies suggest that inflammation may be another factor, either enhancing agent uptake from mucosal sites (46), or expanding the tissue distribution of these agents (47–49). For example, the induction of FDC networks within the ectopic germinal centers of chronically inflamed kidneys enables the accumulation of high levels of PrP<sup>Sc</sup> in this tissue and excretion of low levels of infectivity in urine (47, 48). Likewise, natural chronic inflammatory conditions can expand the deposition of PrP<sup>Sc</sup> in TSE-affected animals, such as to inflamed mammary glands of scrapie-affected sheep (49).

ILFs are recently identified lymphoid structures distributed throughout the intestines of rodents (36, 50–52) and humans (53) with features similar to PPs. Although the small intestine of an adult mouse may contain as many as 100 ILFs, data from the current study and others (30, 37) show that mILFs are highly infrequent (typically <5 per mouse) in tissues from unmanipulated immunocompetent control mice in a specific pathogen-free colony. Stimulation via  $LT\beta R$  is also important for the development of ILFs, but unlike PPs, their formation occurs postnatally (30, 37). In response to exogenous stimuli, LT-expressing B lymphocytes trigger the progression of iILFs into mILFs resembling lymphoid nodules containing a single B lymphocyte follicle of predominantly B2-B lymphocytes with a germinal center and a follicle associated epithelium containing M cells (30, 37, 54). Our analysis also demonstrates that mILFs consistently contain large FDC networks, which are not present in iILFs. Because the inducible nature of mILFs (30, 38) shares features with the ectopically induced germinal centers described above (47–49) we reasoned that mILFs might represent an important novel site of TSE agent accumulation and neuroinvasion in the small intestine.

To specifically determine the potential involvement of mILFs in scrapie agent transmission from the gastrointestinal tract, temporary in utero blockade of the  $LT\beta R$  signaling pathway was used to create mice that lacked PPs (termed PP-deficient mice), but contained numerous mILFs (26, 30). Our experiments demonstrate that FDCs within mILFs are novel and important sites of TSE agent accumulation within the small intestine. Furthermore, due to the presence of a significantly larger number of FDC-containing mILFs in PP-deficient mice, disease susceptibility was unaffected by the absence of PPs. The formation and maturation status of ILFs can be modulated by both luminal bacterial flora (30, 38) and pathogenic microorganisms (39), implying that the microbiologi-

cal status of the intestine at the time of TSE agent exposure might dramatically enhance disease susceptibility.

In the current study, our animals eat the scrapie agent to model oral pathogenesis as closely as possible. Following ingestion of the scrapie agent many factors will act on the inoculum to aid its elimination from the host. Indeed, much of the inoculum will be eliminated from the host by factors including digestion (by enzymes secreted in the stomach or small intestine) and excretion. As a consequence, following oral inoculation very little of the original inoculum will be available to be translocated across the gut epithelium by M cells or other potential mechanisms (41, 42). In WT mice it is likely that once the remaining fraction of the inoculum enters the lamina propria a competitive state exists whereby cells such as macrophages aid the clearance of the TSE agent (55, 56), whereas FDCs act to expand the levels of the TSE agent above the threshold required to achieve neuroinvasion. Thus, in the absence of FDCs (and lymphoid tissue within the intestine) in LT-deficient mice, the residual fraction of the inoculum will be gradually destroyed by phagocytic cells (55, 56). We have previously shown that the temporary depletion of FDCs for ~21–28 days blocks TSE agent transmission from the intestine (13), suggesting this time interval is sufficient to allow clearance of the remaining inoculum. In the current study, no mILFs were detected in the small intestines of  $WT\rightarrow LT\alpha^{-/-}$  mice and  $WT\rightarrow LT\beta^{-/-}$  mice at the time the mice were inoculated with the scrapie agent (5 wk after bone marrow reconstitution) as their formation occurs between 8 and 12 wk after WT bone marrow reconstitution (30, 37). Therefore, by the time the mILFs had eventually developed in the intestines of  $WT\rightarrow LT\alpha^{-/-}$  mice and  $WT\rightarrow LT\beta^{-/-}$  mice, sufficient time will have elapsed to allow clearance of the remaining inoculum, preventing PrP<sup>d</sup> accumulation upon the FDCs within them and subsequent neuroinvasion.

In conclusion, our data demonstrate the crucial importance of TSE agent accumulation upon FDCs within the GALT for efficient neuroinvasion following oral exposure. Current evidence suggests vCJD shares a similar requirement for accumulation upon FDCs before neuroinvasion (5). Thus, our data imply that neuroinvasion of the vCJD agent might also occur soon after accumulation within the GALT before the agent has disseminated to other lymphoid tissues. Our data also identify mILFs as a novel and important site of TSE agent accumulation within the small intestine. As the formation and maturation status of ILFs is strongly influenced by commensal and pathogenic microorganisms (30, 38, 39), factors that stimulate mILF development are likely to dramatically enhance susceptibility to orally acquired TSE agents.

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## Disclosures

The authors have no financial conflict of interest.

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## Role of the draining lymph node in scrapie agent transmission from the skin

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### Abstract

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that affect humans and animals. Diseases include scrapie in sheep and Creutzfeldt–Jakob disease in humans. Following peripheral exposure, TSE agents usually accumulate on follicular dendritic cells (FDCs) in lymphoid tissues before neuroinvasion. Studies in mice have shown that TSE exposure through scarified skin is an effective means of transmission. Following inoculation by this route TSE agent accumulation upon FDCs is likewise essential for the subsequent transmission of disease to the brain. However, which lymphoid tissues are crucial for TSE pathogenesis following inoculation via the skin was not known. Mice were therefore created that lacked the draining inguinal lymph node (ILN), but had functional FDCs in remaining lymphoid tissues such as the spleen. These mice were inoculated with the scrapie agent by skin scarification to allow the role of draining ILN in scrapie pathogenesis to be determined. We show that following inoculation with the scrapie agent by skin scarification, disease susceptibility was dramatically reduced in mice lacking the draining ILN. These data demonstrate that following inoculation by skin scarification, scrapie agent accumulation upon FDCs in the draining lymph node is critical for the efficient transmission of disease to the brain.

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**Keywords:** Transmissible spongiform encephalopathy; Prion disease; Scrapie; Skin; Lymph node; Follicular dendritic cell

### 1. Introduction

Transmissible spongiform encephalopathies (TSEs), or ‘prion diseases’, are sub-acute neurodegenerative diseases that affect humans and both domestic and free-ranging animals. The neuropathological features within the central nervous system (CNS) characteristically include spongiform pathology, neuronal loss, glial activation and amyloid aggregations of an abnormally folded host protein. There is little evidence of pathology in other tissues. The nature of the TSE agent is unknown, but an abnormal, relatively proteinase-resistant isoform ( $\text{PrP}^{\text{Sc}}$ ) of the host cellular prion protein ( $\text{PrP}^{\text{C}}$ ), co-purifies with infectivity in diseased tissues and is a useful biochemical marker [1]. The ‘prion hypothesis’ argues that  $\text{PrP}^{\text{Sc}}$  constitutes a major, or the sole component of infectious agent and facilitates conversion of  $\text{PrP}^{\text{C}}$  to  $\text{PrP}^{\text{Sc}}$  [2].

Many TSE agents, including natural sheep scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in mule deer and elk, and kuru and variant Creutzfeldt–Jakob disease (vCJD) in humans, are acquired by peripheral exposure. Although the main route of transmission of BSE to cattle and other species (e.g.: humans) is considered to be oral (ingestion), other routes of TSE transmission have been identified. In humans accidental iatrogenic transmissions have occurred through transplantation of sporadic CJD contaminated tissues or tissue products, or transfusion of vCJD-contaminated blood [3,4]. Neurosurgical instruments contaminated with TSE agents also have the potential to transmit disease [5]. Therefore the wide tissue tropism of the vCJD agent [6], and potential for large numbers of sub-clinical vCJD cases [7] implies that a serious risk of iatrogenic transmission exists. Studies in mice have also highlighted that skin scarification is an effective means of TSE transmission [8]. Thus, it is possible that some natural TSE cases might be transmitted through skin lesions during close contact, such as from an infected mother to offspring through skin lesions at birth or via the unhealed umbilical cord. Likewise, consumption of coarse feed contaminated with TSE agents

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might also scarify the mucosa of the mouth or oesophagus aiding transmission.

Within the lymphoid tissues of patients with vCJD [6], mule deer fawns with CWD [9], some sheep with natural scrapie [10] and rodents inoculated with scrapie [11] early PrP<sup>Sc</sup> accumulation takes place in the germinal centres upon follicular dendritic cells (FDCs). In the absence of FDCs, TSE agent accumulation in lymphoid tissues and the subsequent spread of disease to the CNS (termed *neuroinvasion*) are both impaired [12–16]. Neuroinvasion from lymphoid tissues proceeds along both sympathetic nerves and fibres of the vagus nerve [17,18].

Following inoculation via skin scarification, the scrapie agent accumulates first in the draining lymph node, and subsequently spreads to non-draining lymph nodes and the spleen [16,19]. TSE agent accumulation upon FDCs in lymphoid tissues is likewise critical for efficient transmission of disease from the skin to the CNS [8,16,20], but which lymphoid tissues are crucial for neuroinvasion is not known. Therefore, in the current study mice deficient in the draining lymph node were utilized to address the following questions: First, whether TSE-susceptibility is reduced in the absence the draining lymph node? Second, whether neuroinvasion occurs from multiple sites such that the lack of the draining lymph node can be compensated for by the presence of another such as the spleen?

## 2. Materials and methods

### 2.1. Mice and bone-marrow grafting

Lymphotoxin (LT) $\alpha^{-/-}$  mice [21] and LT $\beta^{-/-}$  mice [22] were obtained from B & K Universal Ltd. (Hull, UK) and were maintained on a C57BL/6 background. Age- and sex-matched C57BL/6 mice were used as immunocompetent wild-type (WT) controls. Bone-marrow from the femurs and tibias of WT mice was prepared as a single-cell suspension ( $3 \times 10^7$ – $4 \times 10^7$  viable cells/ml) in HBSS (Invitrogen, Paisley, UK). Recipient adult (6–8 weeks old) LT $\alpha^{-/-}$  mice, LT $\beta^{-/-}$  mice and WT mice were  $\gamma$ -irradiated (950 rad) and 24 h later reconstituted with 0.1 ml bone-marrow by injection into the tail vein. Recipient mice were used in subsequent experiments 35 days after bone-marrow grafting. All protocols using experimental rodents were approved by the Institute's Protocols and Ethics Committee and carried out according to the strict regulations of the UK Home Office 'Animals (scientific procedures) Act 1986'.

### 2.2. Scrapie agent inoculation

Mice were inoculated with the ME7 scrapie agent strain by skin scarification of the medial surface of the left thigh. Briefly, prior to scarification approximately 1 cm<sup>2</sup> area of hair covering the site of scarification was trimmed using curved scissors and then removed completely with an electric razor. Twenty-four hours later a 23-gauge needle was used to create a 5 mm long abrasion in the epidermal layers of the skin at the scarification site. Then using a 26-gauge needle one droplet (~6  $\mu$ l) of ME7 scrapie agent inoculum from a 1.0% (w/v) terminal scrapie mouse brain homogenate in physiological saline was

applied to the abrasion and worked into the site using sweeping strokes. The scarification site was then sealed with OpSite (Smith & Nephew Medical Ltd., Hull, UK) and allowed to dry before the animals were returned to their final holding cages. Where indicated, separate groups of WT mice, LT $\alpha^{-/-}$  mice and LT $\beta^{-/-}$  mice were inoculated by intracranial injection with 20  $\mu$ l of a 1.0% (w/v) scrapie mouse brain homogenate (containing approximately  $1 \times 10^{4.5}$  ID<sub>50</sub> units). Following inoculation, all animals were coded, assessed weekly for signs of clinical disease and killed at a standard clinical end-point [23]. Scrapie diagnosis was confirmed by histopathological assessment of TSE vacuolation in the brain.

### 2.3. Immunohistochemical and immunofluorescent analysis

Spleens were removed and snap-frozen at the temperature of liquid nitrogen. Serial frozen sections (10  $\mu$ m in thickness) were cut on a cryostat. Follicular dendritic cells were visualized by staining with mAb 7G6 to detect CD21/CD35 or mAb 8C12 to detect CD35 (BD Biosciences PharMingen, Oxford, UK). B lymphocytes were detected using mAb B220 to detect CD45R (Caltag, Towcester, UK). Complement component (C) C4 was detected using mAb FDC-M2 (AMS Biotechnology, Oxon, UK). For fluorescent microscopy, following the addition of primary antibodies, species-specific secondary antibodies coupled to Alexa Fluor<sup>®</sup> 488 (green) or Alexa Fluor<sup>®</sup> 594 (red) dyes (Invitrogen) were used. Sections were mounted in fluorescent mounting medium (Dako, Ely, UK) and examined using a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK). For light microscopy, following the addition of primary antibody, biotin-conjugated secondary antibodies (Strattech, Soham, UK) were applied followed by alkaline phosphatase coupled to the avidin-biotin complex using Vector Red as a substrate (Vector Laboratories, Peterborough, UK). Sections were subsequently counterstained with hematoxylin to distinguish cell nuclei.

For the detection of PrP in the brain, tissues were fixed in periodate-lysine-paraformaldehyde and embedded in paraffin-wax. Sections (thickness, 6  $\mu$ m) were deparaffinized, and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121 °C, hydration), and subsequent immersion in formic acid (98%) for 5 min [24]. Sections were then stained with the PrP-specific pAb 1B3 [25]. Glial fibrillary acid protein (GFAP) was detected on adjacent brain sections using rabbit GFAP-specific antiserum (Dako). Immunolabelling was detected using either horse radish peroxidase coupled to the avidin-biotin complex (Vector Laboratories) with diaminobenzidine (DAB) as a substrate, or alkaline phosphatase coupled to the avidin-biotin complex using Vector Red as a substrate. Sections were subsequently counterstained with hematoxylin to distinguish cell nuclei.

### 2.4. Immunoblot detection of PrP<sup>Sc</sup>

Spleen fragments (approximately 20 mg) or inguinal lymph nodes (ILNs) were prepared as 10% (w/v) tissue homogenates and PrP<sup>Sc</sup> enriched by sodium phosphotungstic acid (NaPTA)



precipitation [26] and treated in the presence or absence of proteinase K (40 µg/ml, 60 min, 37 °C; VWR, Lutterworth, UK). Following enrichment, pellets were re-suspended and diluted to an approximate protein concentration of 0.5 mg protein/ml and 10 µl electrophoresed through SDS/PAGE (12%) polyacrylamide gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hemel Hemstead, UK) by semidry blotting. PrP was detected with the PrP-specific mouse mAb 8H4 [27], followed by horse radish peroxidase-conjugated goat anti-mouse antiserum. Bound horse radish peroxidase activity was detected with Supersignal® West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL, USA).

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Significant differences between samples in different groups were sought by one-way ANOVA.

3. Results

3.1. Creation of mice that lack inguinal lymph nodes

In mice the lymph nodes that drain the skin region of the thigh are the inguinal lymph nodes (ILNs), and our previous studies have shown that the TSE agent accumulates first in these lymph nodes following inoculation via scarification of the skin of the thigh [16,19]. To test the hypothesis that the draining lymph node plays an important role in TSE agent transmission from the skin to the CNS, mice were created that lacked the draining ILNs, but had mature FDCs in remaining lymphoid tissues such as the spleen. Stimulation through the LTβ-receptor (LTβR) during embryonic development is important for the formation of most lymph nodes but not the spleen [28]. As a consequence the ILNs are lacking in LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice (Fig. 1A). However, post-natal LTβR-stimulation is also critically required to induce the maturation of FDCs and maintain them in their differentiated state [29]. As a consequence, the remaining spleens of LTα<sup>-/-</sup> mice (Fig. 1B) and spleens (Fig. 1B) and mesenteric lymph nodes (data not shown) of LTβ<sup>-/-</sup> mice are deficient in FDCs. Since FDCs are critical for the efficient neuroinvasion of

the ME7 scrapie agent following inoculation via the skin [16,20], it was important therefore to induce the development of FDCs in the remaining lymphoid tissues in LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice.

Adult WT mice, LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice were γ-irradiated and 24 h later reconstituted with LT-expressing WT bone marrow (termed WT → WT mice, WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice, respectively). Within 35 days of WT bone marrow transfer, the development of FDC networks in the remaining lymphoid tissues of LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice was induced (Fig. 1B), but the development of missing lymphoid tissues was not induced (Table 1; [30]). Expression of complement receptors (CR)1 and CR2 (CD35 and CD21, respectively) by FDCs within the spleens of WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice appeared similar to that observed in tissues from immunocompetent control WT → WT mice (Fig. 1B). The close association of complement component C4 with the CR1-expressing cells indicated that the spleens of WT → WT mice, WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice contained functional, immune complex-trapping FDCs (Fig. 1C).

3.2. Effect of ILN-deficiency on scrapie pathogenesis

Groups of WT → WT mice, WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice were inoculated with the scrapie agent via skin scarification 35 days after reconstitution with WT bone marrow. All immunocompetent WT → WT mice succumbed to clinical TSE disease with a mean incubation period of 322 ± 9 days (n = 9, Table 1). In contrast, in the absence of the draining ILNs, scrapie-susceptibility was dramatically reduced. Six of seven WT → LTα<sup>-/-</sup> mice remained free of the signs of scrapie up to 503 days after inoculation (Table 1). However, one WT → LTα<sup>-/-</sup> mouse did succumb to clinical scrapie with an incubation period of 343 days. Likewise, six of eight WT → LTβ<sup>-/-</sup> mice remained free of the signs of scrapie up to 503 days after inoculation (Table 1). Characteristic spongiform pathology, disease-specific abnormal accumulations of PrP (PrP<sup>d</sup>) and reactive astrocytes expressing high levels of GFAP were detected within the brains of all WT → WT mice, and the few WT → LTα<sup>-/-</sup> mice (n = 1) and WT → LTβ<sup>-/-</sup> mice (n = 2) that developed clinical signs of scrapie (Fig. 2). In contrast, spongiform pathology, PrP<sup>d</sup> accumulation and reac-

Table 1  
Scrapie agent transmission following inoculation via skin scarification is dramatically reduced in mice lacking the draining inguinal lymph node

Mouse strain <sup>a</sup>	Presence at time of inoculation				Disease incidence	Mean incubation period (days) ± S.E.M.
	ILNs	MLNs	SPL	FDCs		
WT → WT	+	+	+	+	9/9	322 ± 9
WT → LTα <sup>-/-</sup>	–	–	+	+	1/7	343, 5X > 503 <sup>b</sup>
WT → LTβ <sup>-/-</sup>	–	+	+	+	2/8	334, 350 6X neg. 403–503 <sup>c</sup>

ILNs, inguinal lymph nodes; MLNs, mesenteric lymph nodes; SPL, spleen; FDCs, follicular dendritic cells.  
(+) present; (–) absent; neg., mice free of the clinical and histopathological signs of scrapie at the time of analysis.  
<sup>a</sup> Mice were inoculated with the scrapie agent 35 days after γ-irradiation and reconstitution with WT bone marrow.  
<sup>b</sup> Two additional mice were killed 336 and 432 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull. No histopathological signs of scrapie were detected in the brain (data not shown).  
<sup>c</sup> Six mice were killed 403, 406, 476, 490 and 503 days after inoculation. These mice were free of the clinical signs of scrapie at the time of cull. No histopathological signs of scrapie were detected in the brain (data not shown).



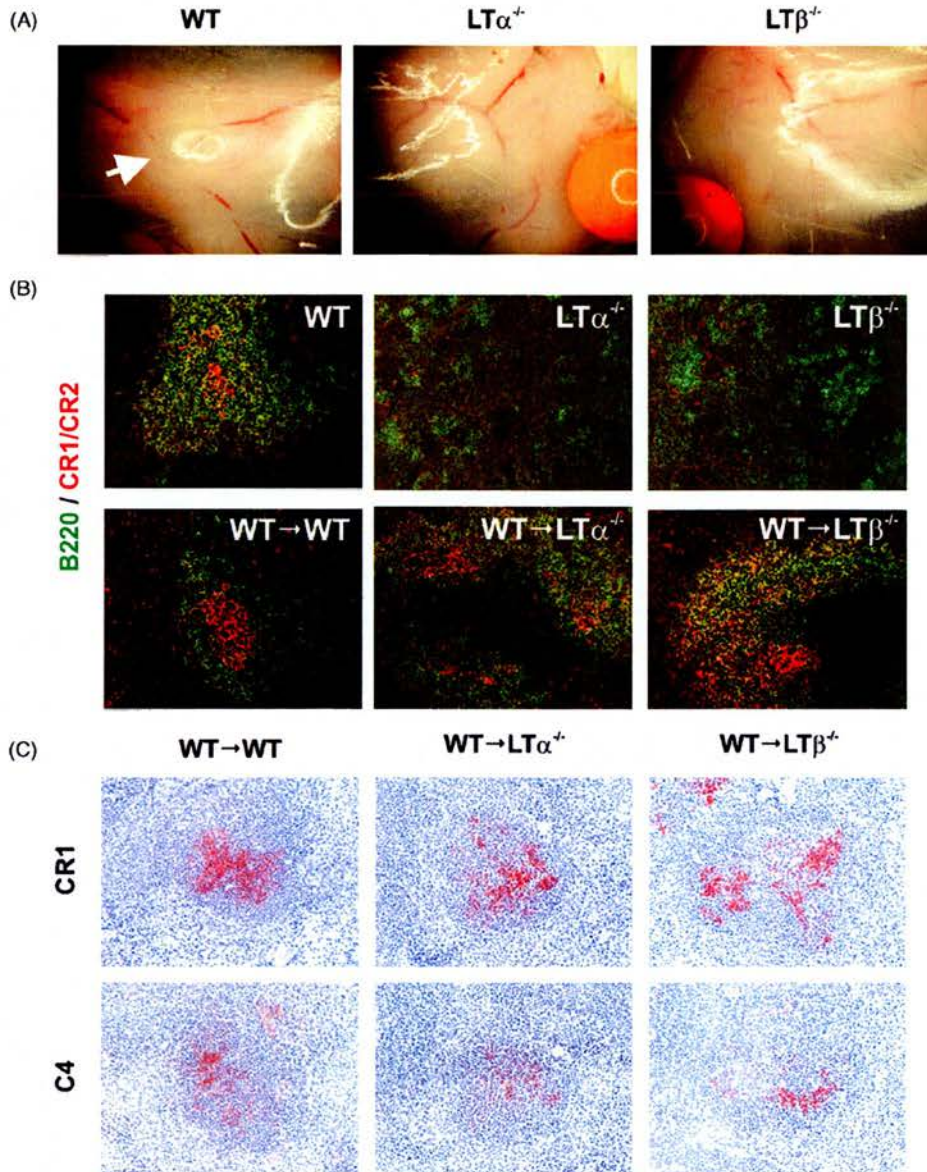


Fig. 1.  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice lack ILNs and FDCs in remaining lymphoid tissues. (A) In contrast to WT mice (left, arrow),  $LT\alpha^{-/-}$  mice (middle) and  $LT\beta^{-/-}$  mice (right) lack ILNs. (B and C) Immunohistochemical analysis of spleen microarchitecture. (B) Spleens from WT mice and WT  $\rightarrow$  WT mice showed presence of CR1/CR2-expressing (red) FDCs and organised B lymphocyte follicles (B220-positive cells; green). FDCs and distinct B lymphocyte follicles were undetectable in the spleens of unreconstituted  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice. After reconstitution of  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice with WT bone marrow (WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice, respectively), FDC maturation and follicular organisation were induced. (C) Spleens from WT  $\rightarrow$  WT mice, WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice contain functional FDCs. Complement component C4 (red; lower row) was detected in close association with CR1-expressing FDCs (red; upper row). Original magnification in panels B and C,  $\times 200$ .

tive astrocytes were not detected in the brains of any of the WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice that were free of the clinical signs of scrapie (Fig. 2).

When inoculated with the scrapie agent directly into the CNS (by intracranial injection) all WT mice,  $LT\alpha^{-/-}$  mice and  $LT\beta^{-/-}$  mice developed clinical signs of scrapie with similar incubation periods of 172–175 days ( $n=6$ ; data not shown). Thus, the dramatically reduced susceptibility of WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice to the scrapie agent when inoculated via the skin could not be attributed to a role for  $LT\beta$  signalling in disease pathogenesis directly within the CNS.

### 3.3. $PrP^{Sc}$ accumulation in remaining lymphoid tissues

Strong accumulations of relatively proteinase K-resistant  $PrP^{Sc}$  were found in both the ILNs and spleens of WT  $\rightarrow$  WT mice (Fig. 3A and B). Although WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice lack ILNs, the area of subcutaneous tissue where this lymph node would have been located in WT mice was removed and  $PrP^{Sc}$  levels within it determined. Congruent with the absence of the ILNs,  $PrP^{Sc}$  was likewise undetectable in subcutaneous tissue taken from WT  $\rightarrow$   $LT\alpha^{-/-}$  mice and WT  $\rightarrow$   $LT\beta^{-/-}$  mice that remained free of clinical disease up to

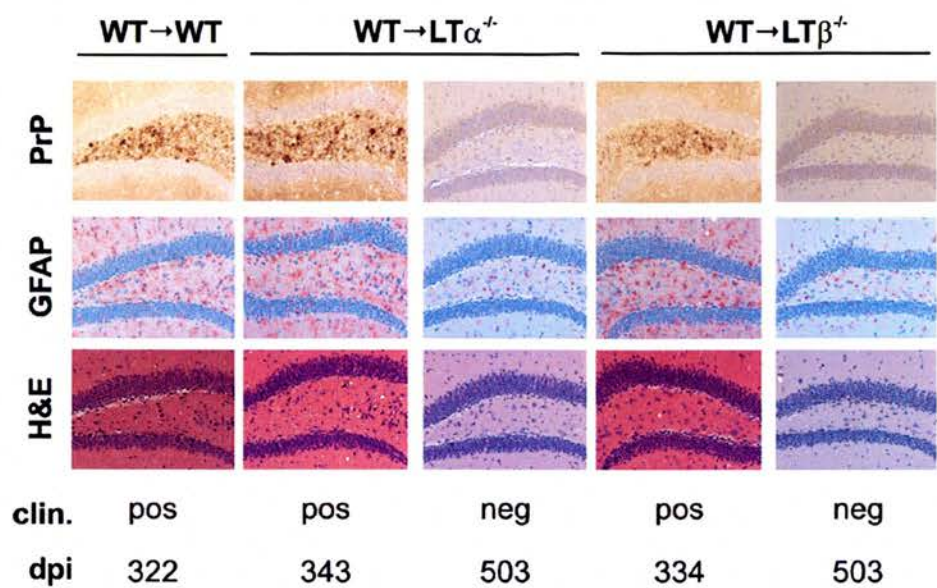


Fig. 2. Immunohistochemical analysis of brain tissue from terminally scrapie affected mice and mice that remained free of the signs of disease at the end of the experiment. Large PrP<sup>d</sup> accumulations (brown; upper row), reactive astrocytes expressing high levels of GFAP (red; middle row) and spongiform pathology (H and E; lower row) were detected in the hippocampi of all clinically scrapie-affected WT→WT mice, and the few WT→LTα<sup>-/-</sup> mice (n = 1) and WT→LTβ<sup>-/-</sup> mice (n = 2) that developed clinical disease. None of these pathological signs of scrapie were detected in any of the brains from scrapie-inoculated WT→LTα<sup>-/-</sup> mice (n = 6) and WT→LTβ<sup>-/-</sup> mice (n = 6) that remained free of the clinical signs of disease at the end of the experiment. All sections were counterstained with haematoxylin (blue). Original magnification ×200. dpi, day post inoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie.

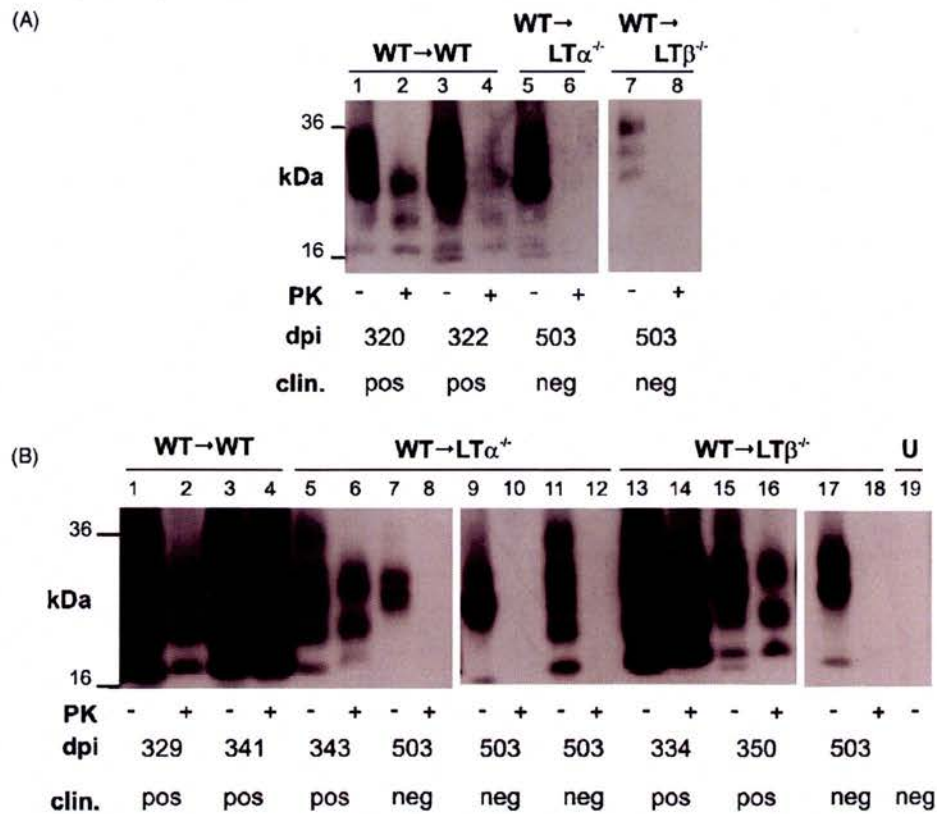


Fig. 3. PrP<sup>Sc</sup> accumulation in the ILNs (A) and spleens (B) of WT→WT mice, WT→LTα<sup>-/-</sup> mice and WT→LTβ<sup>-/-</sup> mice inoculated with the scrapie agent by skin scarification. Samples were treated in the presence (+) or absence (–) of proteinase K (PK) prior to electrophoresis. After PK treatment, a typical three-band pattern was observed between molecular mass values of 20 and 40 kDa, representing unglycosylated, monoglycosylated and diglycosylated isomers of PrP (in order of increasing molecular mass). Lane U, spleen from an uninoculated control WT mouse; dpi, day post inoculation on which the tissues were analyzed; pos, mice that developed clinical signs of scrapie; neg, mice that were free of the signs of scrapie.



503 days after inoculation (Fig. 3A). Similarly, PrP<sup>Sc</sup> was also undetectable in the spleens of clinically negative WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice (Fig. 3B). In contrast, in the few WT → LTα<sup>-/-</sup> mice (*n* = 1) and WT → LTβ<sup>-/-</sup> mice (*n* = 2) that developed clinical signs of scrapie, strong accumulations of PrP<sup>Sc</sup> were detected in their spleens (Fig. 3B; lanes 6, 14 and 16, respectively).

#### 4. Discussion

We show here that the draining lymph node plays a crucial role in TSE agent neuroinvasion following inoculation via the skin. Our data show that in the absence of the draining lymph node at the time of inoculation, transmission of the scrapie agent from the skin is dramatically reduced, despite the presence of mature FDCs within the spleen and other remaining lymphoid tissues. Thus following inoculation via the skin TSE agent accumulation upon FDCs in the draining lymph node is critical for efficient neuroinvasion. We have previously shown that following inoculation via scarification of skin of the thigh, the TSE agent accumulates first within the draining ILN and subsequently accumulates within the spleen [16,19]. However, data here show that the spleen is not an important site of neuroinvasion as mature FDCs were present within the spleens of WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice that remained refractory to clinical disease. Together, these data demonstrate that TSE agent accumulation within the draining lymph node is critically required for efficient neuroinvasion following inoculation via scarification of the skin.

Stimulation through the LTα<sub>1</sub>β<sub>2</sub>-LTβR signalling pathway during embryonic development is important for the formation of most lymph nodes but not the spleen [28]. As a consequence the ILNs and most other lymph nodes do not form in LTα<sup>-/-</sup> mice or LTβ<sup>-/-</sup> mice [28]. In adult mice, LTβR-stimulation is also critically required to induce the maturation of FDCs and maintain them in their differentiated state [29]. Thus, LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice lack most lymph nodes and FDCs in their remaining lymphoid tissues. Reconstitution of LTα<sup>-/-</sup> mice and LTβ<sup>-/-</sup> mice with WT bone marrow induces the development of FDC networks in the remaining lymphoid tissues, but does not restore the development of the missing lymphoid tissues (Table 1; [30]). FDCs are critical for the efficient neuroinvasion of the ME7 scrapie agent following inoculation via the skin as disease susceptibility is reduced in their absence [16,20]. Therefore in the current study through reconstitution of LTα<sup>-/-</sup> mice or LTβ<sup>-/-</sup> mice with WT bone marrow we created mouse models that lacked the draining ILNs but had mature FDC networks in the remaining lymphoid tissues. In these models, the presence of FDCs in the remaining tissues would enable TSE agent accumulation to occur within them if the ILNs were not required for efficient neuroinvasion.

FDCs are a distinct lineage from the migratory bone marrow-derived dendritic cells (DCs). FDCs are considered to derive from stromal precursor cells, are tissue fixed and do not phagocytose antigens. Instead, FDCs characteristically trap and retain immune-complexes composed of antigen, antibody and/or complement components, storing them on their surfaces in native

form for long durations [31]. Immune complex retention on FDCs is highly dependent on complement components and the expression of CR1 and CR2 [32]. The expression of CR1 and CR2 by FDCs within the spleens of WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice was similar to that observed in tissues from immunocompetent control WT → WT mice. Furthermore, the close association of complement component C4 with the CR1-expressing cells implied that 35 days after bone marrow transfer the remaining lymphoid tissues of WT → WT mice, WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice contained functional immune complex trapping FDCs. Indeed, previous studies have demonstrated that reconstitution of LTα<sup>-/-</sup> mice with WT bone marrow or lymphocytes induces the development of FDC networks in the spleen which support immunoglobulin (Ig) class switching and maintain an effective IgG response [33,34]. Current hypotheses suggest that complement plays an important role in the localisation of TSE agents to FDCs [35,36]. Thus these data indicate that the FDCs within the remaining lymphoid tissues of WT bone marrow-reconstituted mice had the potential to acquire the scrapie agent.

Despite the presence of mature FDC networks within the remaining lymphoid tissues of WT → LTα<sup>-/-</sup> mice (spleen and nasal associated lymphoid tissue) and WT → LTβ<sup>-/-</sup> mice (spleen, cervical lymph nodes, mesenteric lymph nodes and nasal associated lymphoid tissue), scrapie susceptibility following inoculation by skin scarification was dramatically reduced. Similarly, in the spleens of WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice that remained free of the signs of clinical disease the accumulation of PrP<sup>Sc</sup> was blocked. Although we have previously demonstrated that TSE agent accumulation upon FDCs is critical for the efficient transmission of disease from the skin to the brain the precise tissue within which neuroinvasion occurs was not known [16]. Data in the current study imply that the initial neuroinvasion occurs following accumulation upon FDCs within the draining lymph node.

We cannot entirely exclude the possibility that the ILNs serve as the initial site of scrapie agent accumulation, with neuroinvasion occurring following subsequent dissemination to other lymphoid tissues. However, data from other studies also implies that the major route of neuroinvasion following inoculation via the skin is not via the spleen. Removal of the spleen before subcutaneous inoculation with the scrapie agent has no effect on disease pathogenesis [37]. The temporary depletion of FDCs prior to inoculation with the scrapie agent via the skin significantly reduces disease susceptibility [16]. However, the lack of any observable effect of such treatment at a time when heavy accumulations of PrP<sup>Sc</sup> are detectable in the draining ILNs but not the spleen or non-draining ILNs [16] indicates that neuroinvasion most likely occurs at a very early stage post exposure and before the agent has had sufficient time to disseminate to multiple lymphoid tissues.

In the current study, every effort was taken at the time of skin scarification to avoid drawing blood to prevent the leakage of infectivity directly into the bloodstream during inoculation. If the scrapie agent had leached into the bloodstream a need for the draining ILNs in neuroinvasion would obviously have been by-passed. As a consequence, all WT → LTα<sup>-/-</sup>



mice and WT → LTβ<sup>-/-</sup> mice would be fully susceptible to disease as scrapie agent neuroinvasion could have occurred via the spleen which filters antigen and microorganisms from the blood-stream. However, most WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice remained free of clinical disease and PrP<sup>Sc</sup> was undetectable within the spleens of these animals. These data clearly demonstrate that the major route of scrapie transmission from the skin to lymphoid tissues does not occur via the bloodstream. We have previously demonstrated that PrP<sup>Sc</sup> accumulates first upon FDC networks within the draining ILN and subsequently spreads to non-draining lymph nodes and the spleen [16]. Therefore, taken together these data indicate that following inoculation via the skin the scrapie agent is first propagated to the draining lymph node via the lymphatics, and subsequently distributed to the spleen and other lymphoid tissues via the bloodstream. However, one WT → LTα<sup>-/-</sup> mouse and two WT → LTβ<sup>-/-</sup> mice did develop clinical scrapie despite the absence of the draining ILN. Although the precise route of neuroinvasion in these rare instances is uncertain, we can not exclude the possibility that neuroinvasion occurred following contamination of the blood-stream or via direct infection of peripheral nerves at the site of skin scarification, by-passing the need for accumulation within the draining ILN.

The levels of infectivity responsible for natural TSE transmissions are unknown but are likely to be much lower than the moderate dose used in this study. The lack of detection of PrP<sup>Sc</sup> within the spleens and brains of surviving WT → LTα<sup>-/-</sup> mice and WT → LTβ<sup>-/-</sup> mice provides strong evidence that scrapie transmission was blocked in the absence of the draining ILN. However, these data do not exclude the possibility that following inoculation with a much higher dose of scrapie than the one used here neuroinvasion might occur via an alternate route, such as direct uptake by peripheral nerves.

Data presented here show that draining lymphoid tissue local to the site of inoculation (ILNs in the current study) is critically required for the efficient transmission of the ME7 scrapie agent following inoculation via skin scarification. Together, our data suggest that following inoculation through this route neuroinvasion occurs via the following pathway. Following inoculation into the skin, the TSE agent disseminates first to the draining lymph node via the lymph. The precise mechanism by which the TSE agent is transported within the draining afferent lymph remains to be identified [19]. Neuroinvasion does not occur following direct uptake by peripheral nerves in the skin or following widespread dissemination via the blood-stream [8,20]. Within the draining lymph node, TSE agent accumulation occurs upon PrP<sup>C</sup>-expressing FDCs [20]. Accumulation upon FDCs is critical for efficient neuroinvasion [16]. Following accumulation within the draining lymph node the TSE agent is subsequently disseminated to non-draining lymph nodes and the spleen most likely via the blood-stream [16,19]. Data in the current study demonstrate that neuroinvasion is dramatically impaired in the absence of the draining lymph node implying that the TSE agent most likely spreads to the peripheral nervous system directly from this tissue.

Current evidence suggests that the vCJD [6,38,39], CWD [9] and sheep scrapie [10] agents share a similar requirement for

accumulation upon FDCs prior to neuroinvasion. Thus the initial neuroinvasion of naturally-acquired TSE agents might also occur following accumulation within the draining lymphoid tissue and before the agent has disseminated to other lymphoid tissues. TSE agents cause considerable neurodegeneration once they infect CNS. Understanding the early events in TSE agent neuroinvasion from peripheral tissues will be important for the development of therapeutic strategies to combat these fatal neurodegenerative diseases.

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